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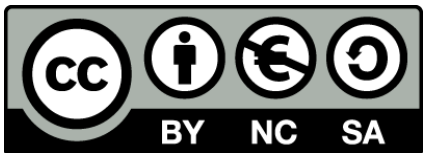
Mechanisms of restricted activation of the Torso receptor: from the eggshell to the embryo

Alessandro Mineo



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TESIS DOCTORAL

Mechanisms of restricted activation of the Torso receptor: from the eggshell to the embryo

Memoria presentada por

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Para optar al grado de

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Mi relato será fiel a la realidad o, en todo caso, a mi recuerdo personal de la realidad, o cual es lo mismo.

Jorge Luis Borges
El Libro de Arena

A mia madre, mio padre,

e Fedi

CONTENTS

Introduction

1.1 Symmetry breaking in embryonic development

The development of a multicellular organism from a single egg cell requires both the differentiation of many cell types and the organisation of these cells into patterns. The formation of well-defined structures and patterns at both microscopic and macroscopic scales, in a fecundated oocyte, requires multiple "symmetry breaking" events (Anderson, 1972). These events are intimately linked to functional diversification on every scale, from molecular assemblies, to subcellular structures, to cell types themselves, tissue architecture and embryonic body axes. Different cellular signaling mechanisms have been identified as responsible for triggering several cellular responses that, in turn, generate, cellular diversity. However, cell signalling molecules have to be restricted to specialised sub-populations of cells in order to break locally the uniformity.

Although the symmetry breaking modalities identified so far are achieved in the most diverse ways, in many cases, the asymmetric fate of a system appears to follow from certain "cues" that pre-exist before symmetry is broken. These cues are either localised signals that come from the extracellular milieu, or "landmarks" (Li and Bowerman, 2010).

The development of the body axes of the Drosophila embryo constitutes a model system to study symmetry breaking events that coordinate differentiation of specialised structures along the body. As the embryo develops as a syncytium for the early embryogenesis stages, the local asymmetry that allows differentiation of specific body parts relies on cues that were generated in oogenesis together with the oocyte.

In this thesis, we focused on the mechanisms by which the asymmetry, generated by spatial cues during oogenesis, is, then, supplied to the embryo to ensure local restricted activation of signaling molecules that give rise to the embryonic axes.

1.2 Drosophila life cycle

Drosophila melanogaster is a holometabolous insect undergoing a complete metamorphosis. At 25ºC its life cycle last ten days. Soon after being deposited by the female, the zygote starts dividing and start embryogenesis into the egg. This stage lasts for one day. During this time, the embryo develops into a larva (first instar larva or L1)

that hatches out of the egg, crawls into a food source, and eats until reaching the molt. After a day, the first instar larva molts and becomes the second instar larva (or L2) and, after one day in this stage, the larva molts again to become the third instar larva (or L3). After two days of eating in this stage, the larva crawls out of the food source and enters a quiescent state (prepupa) and starts metamorphosis (pupa). *Drosophila* stays in the pupal stage for about five days. During this time, metamorphosis takes place replacing larval tissues with the adult ones. When the adults emerge from the pupa, they are fully formed and fertile.

Since the specification of embryonic axes in *Drosophila* is a direct consequence of asymmetries generated in oogenesis, thus, we will discuss the main events occurring in oogenesis.

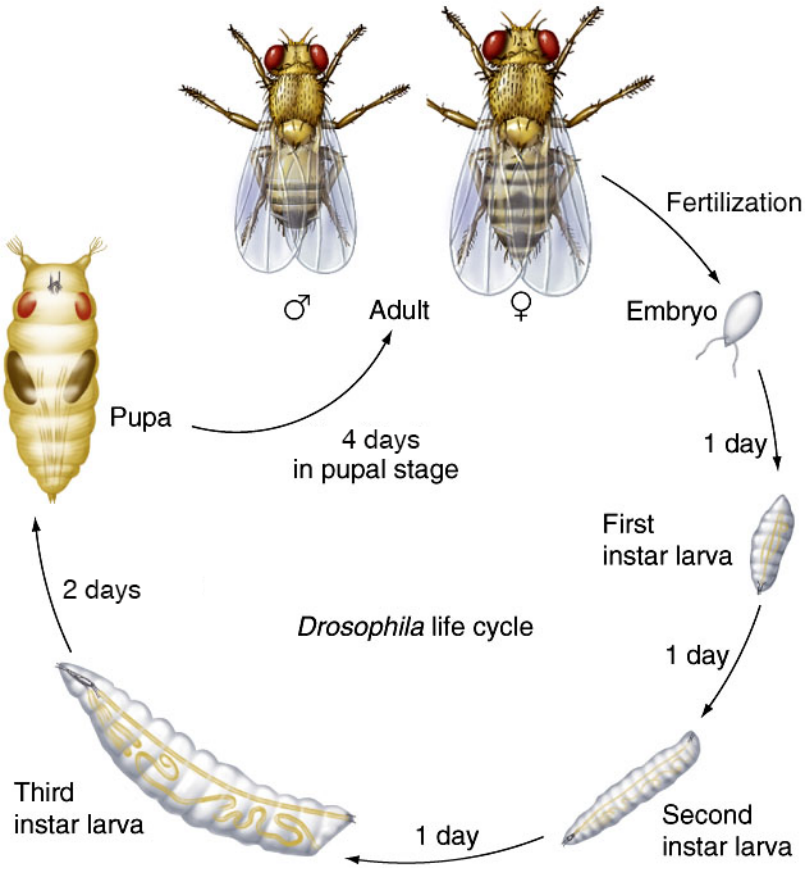

Figure.1 *Drosophila melanogaster* life cycle at 25 °C (adapted from Flybase).

1.3 Oogenesis

Females flies have two ovaries, each of them containing 16-20 ovarioles. Each ovariole contains a series of developing egg chambers divided into 14 stages according to morphological criteria outlined in Figure 2 (Cavaliere et al., 2008; King, 1970). Each egg chamber is constituted by the oocyte and the nurse cells surrounded by the somatic follicular epithelium (King, 1970; Spradling, 1993). Each ovariole constitute an individual egg assembly line, where younger egg chambers are located in the anteriormost region and progressively older egg chambers are located posteriorly. The most anterior region of the ovariole is called the germarium, where somatic and germline components of the follicle originate and come together to create the basic structure of the egg chamber (Spradling, 1993). Once the egg chamber goes out of the germarium, it enters the posterior region of the ovariole called the vitellarium where the oocyte progressively increases its size.

Egg chambers arise at the tip of each ovariole, in the germarium where germ stem cell division takes place (Lin and Spradling, 1993). Germ line stem cells divide giving rise to another stem cell and to a cystoblast which in turn divides four times giving rise to a cyst composed of 16 cells interconnected by cytoplasmic bridges, known as ring canals (Robinson et al., 1994). Among the 16 cells, only one of them will become the oocyte whereas the others will become nurse cells that will provide the oocyte with nutrients, proteins and mRNAs through the ring canals. Each cyst is surrounded by epithelial monolayer of somatic follicle cells derived from the ovarian mesoderm that are important for patterning the oocyte, the production of yolk proteins and the secretion of the eggshell proteins (Spradling, 1993).

As the cyst progresses into the germarium, the future oocyte is positioned posteriorly and the whole cyst becomes incapsulated by follicle cells arising from somatic stem cells located in the germarium. Once the cyst is enveloped, follicle cells migrate at the anterior and posterior of the cyst separating from its younger and older neighbours respectively. Follicle cells that encapsulate the germline cyst give rise to different cell types: the polar cells (a pair of follicle cells with round morphology at each end of the egg chamber), stalk cells (specialised follicle cells that form short stalks that interconnect individual egg chambers) and the follicle cells that form the columnar epithelium that covers the cyst (Horne-Badovinac and Bilder, 2005; Lopez-Schier,

2003).

From the encapsulation of the cyst until the beginning of vitellogenesis (stage 6-7), follicle cells proliferate and form a cuboidal epithelium. During vitellogenesis, the oocyte grows and follicle cells reorganise and come into contact with the oocyte in order to secrete the eggshell components (Horne-Badovinac and Bilder, 2005). At the beginning of stage 9, follicle cells migrate posteriorly to cover the oocyte undergoing from a cuboidal to a columnar transition (Horne-Badovinac and Bilder, 2005). At the same time, a group of follicle cells localised anteriorly, named border cells, delaminate and start migrate posteriorly through the nurse cells towards the oocyte (Fig.3) (Montell et al., 2012).

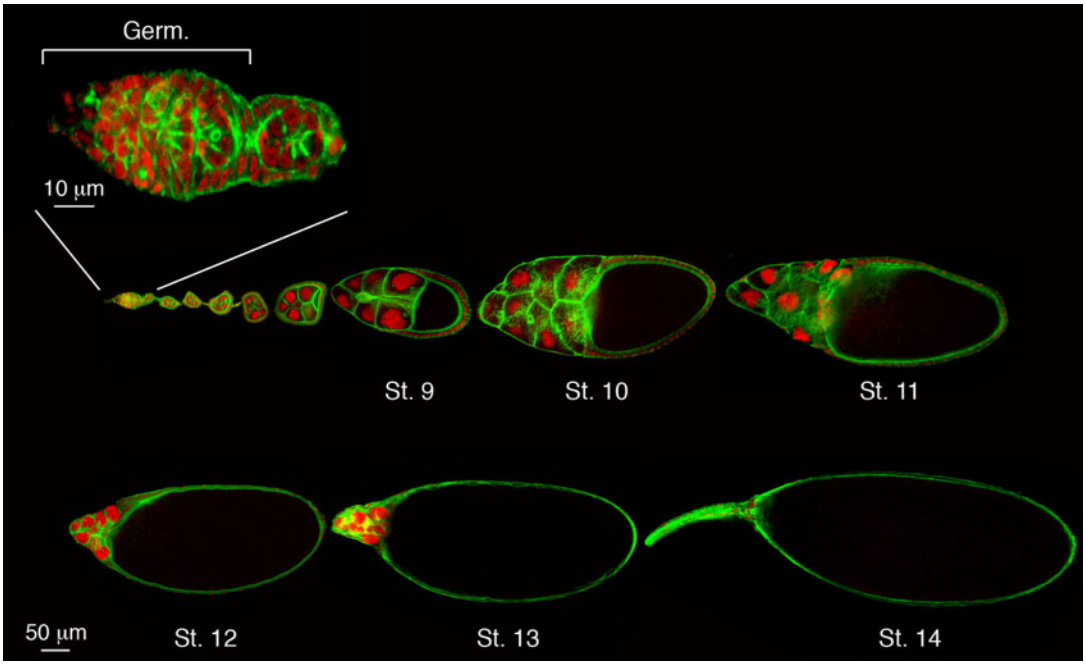

Figure.2 Overview of oogenesis. Confocal cross-sections of egg chambers stained with FITC-phalloidin (green) labelling the F-actin cytoskeleton and Propidium Iodide (red) labelling the nuclei. In the top left, magnification of the gemraarium and stage 1 egg chamber. During mid and late oogenesis (stages 9–14), follicle cells secrete the component of the eggshell and the oocyte dramatically increase in size (adapted from Cavaliere et al., 2008).

At stage 10B, once border cells have reached the oocyte, antero-lateral follicle cells, named centripetal cells, migrate towards the anterior of the oocyte (Fig.3) (Montell et al., 2012). In this way, the oocyte is entirely surrounded by follicle cells that start to

secrete and build the eggshell. By the end of stage 10B, nurse cells contract to extrude their content into the oocyte and, subsequently, shrink and start degenerating by apoptosis.

The oocyte sends signals to the surrounding follicle cells organising the antero-posterior and dorso-ventral axes of the egg chamber. Indeed, around stage 6, the oocyte is already positioned posteriorly to the nurse cells and its nucleus is located in a posterior region. The oocytes nucleus controls the export of the *gurken* (*grk*) mRNA that encodes a member of the EGFR (Epidermal Growth Factor Receptor) ligand family (Neuman-Silberberg and Schupbach, 1993).

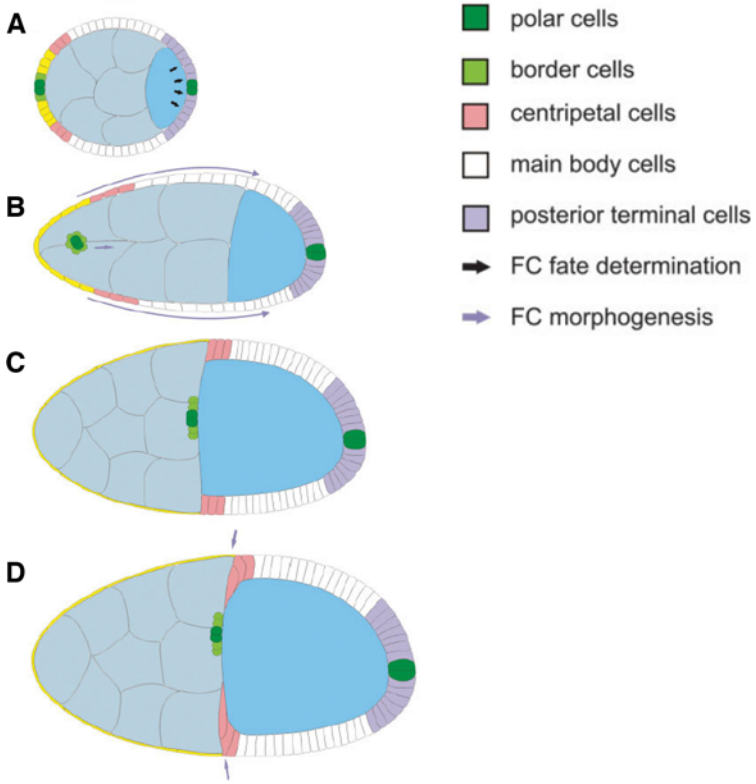

Figure. 3 Patterning and morphogenesis of the follicular epithelium (A) The *gurken* signal from the oocyte instructs the posterior terminal cells to adopt a different fate from their anterior counterparts. (**B–D**) Follicle cell migrations to surround the oocyte. (**B**) During stage 9, the majority of the follicle cells migrate toward the posterior of the egg chamber. (**C**) At stage stage 10A, the migration is completed. **D**) At stage 10B, the centripetal cells migrate laterally between the nurse cells and oocyte to completely enclose the anterior of the oocyte together with the border cells. (adapted from Horne-Badovinac and Bilder, 2005).

Tranlastion and secretion at very short range of the Grk protein induce the activation of the EGFR (Torpedo) in adjacent follicle cells, that acquire a “posterior fate” (Fig.4) (Gonzalez-Reyes et al., 1995; Roth et al., 1995). Posterior follicle cells respond secreting a signal that reorganise the entire oocyte microtubule cytoskeleton promoting the migration of the nucleus from the posterior to an antero-lateral position (Gonzalez-Reyes and St Johnston, 1994; Poulton and Deng, 2007). Here, the nucleus induces a second wave of Grk leading to the “dorsal” specification of adjacent follicle cells and restricting the expression of *pipe* in ventral follicle cells leading to the specification of the future embryonic dorso-ventral axis (Fig.4) (Neuman-Silberberg and Schuepbach 1993; Gonzalez-Reyes et al., 1995; Roth et al., 1995).

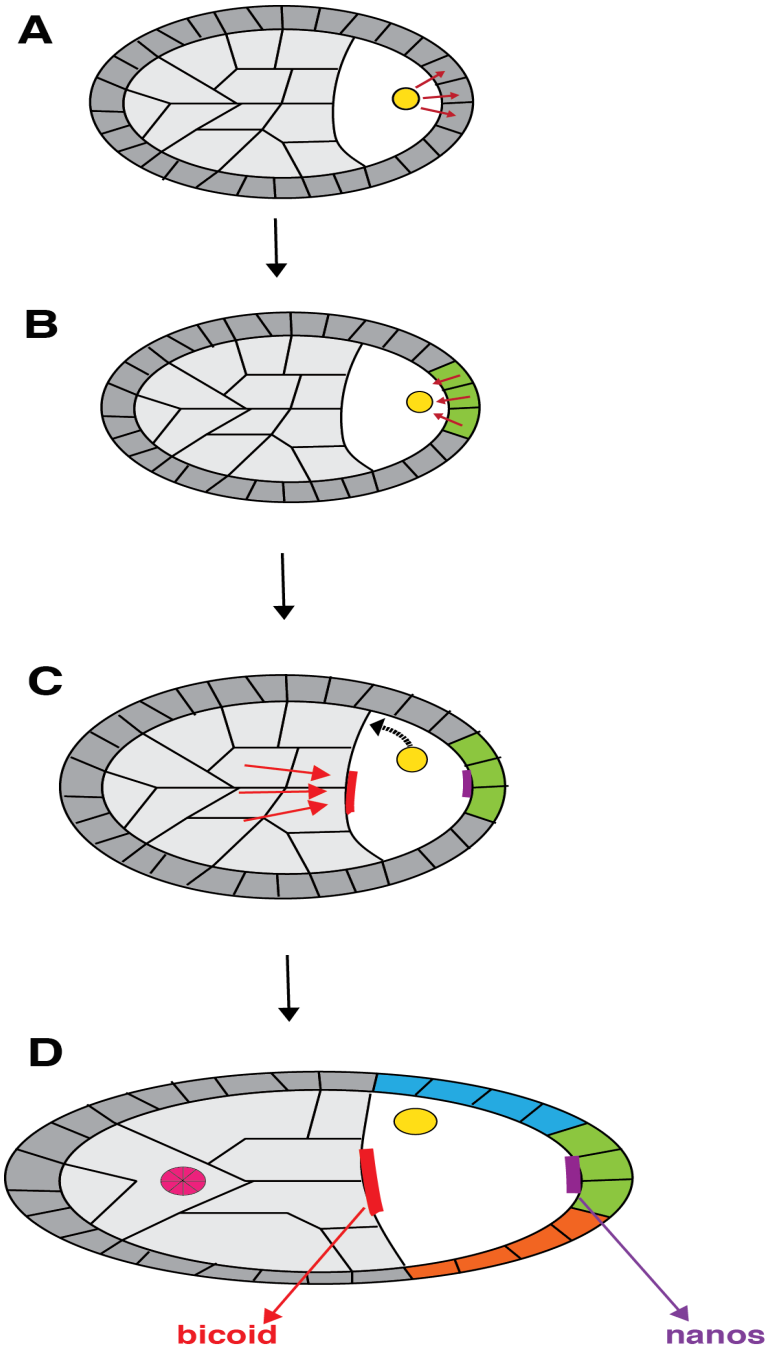

Figure.4 Specification of follicle cells popupations in the egg chamber. (**A**) At stage 6, the oocyte nucleus secretes Grk in adjacent follicle cells, that acquire a "posterior fate" (in green). (**B**) Posterior follicle cells respond secreting a signal that (**C**) reorganise the entire microtubule cytoskeleton promoting the migration of the nucleus from the posterior to an antero-lateral and the localisation of the anterior (*bicoid*) and posterior (*nanos*) determinants at the poles (arrows). (**D**) At stage 8, the nucleus induces a second wave of Grk leading to the "dorsal" specification of adjacent follicle cells (in light blue) and restricting the expression of *pipe* in ventral follicle cells (in orange).

At the same time, around stage 8, nurse cells produce mRNAs that will specify the anterior and posterior axes, *bicoid* and *nanos* respectively. These mRNAs are transported to the oocyte where they localise to the anterior (*bicoid*) and posterior (*nanos*) poles remaining translationally inactive until early embryogenesis (Fig.4) (Steinhauer and Kalderon, 2006).

1.4 Late oogenesis and the formation of the eggshell

The eggshell is constituted by five morphologically distinct layers: vitelline membrane, wax layer, inner chorion layer, endochorion and exochorion (Margaritis et al., 1980 (Fig. 5).

The vitelline membrane is the innermost eggshell layer and the first one in being secreted by follicle cells. Its deposition begins at stage 9 and is completed by late stage 10 (Waring, 2000). At stage 9, vitelline membrane proteins are secreted and accumulated in the space between the oocyte and the follicle cells in small vesicles, called vitelline bodies, that remain separated by microvilli, membrane protrusions extending from the oocyte (Trougakos et al., 2001). At stage 10B, microvilli shorten and vitelline bodies fuse to form a continuous layer that covers the oocyte (Margaritis et al., 1980; Margaritis, 1985)

At the end of stage 10, it starts the deposition of the wax layer with the accumulation of lipid-filled vesicles between follicle cells and vitelline membrane. Lipid vesicle are synthesised and accumulate on the surface of the vitelline membrane until stage 12 (Papassideri et al., 1991; Papassideri et al., 1993). In late stage 12 until stage14, chorion proteins start to be produced and secreted. The inner endochorion layer (ICL) is deposited, through secretory vesicles from the follicle cells, on the wax layer surface (Papassideri et al., 1993). Progressive secretion of proteins increases gradually the thickness of the layer, whereas other proteins are subsequently secreted to give rise to the following chorion layers (Papassideri and Margaritis, 1996). During the initial stages of choriogenesis (12-13), the ICL forms a bilaminar complex, while by stage 14, it separates forming a 3-dimensional crystal (Papassideri and Margaritis, 1996). Endochorion and exochorion are produced at stage 14. Ultrastructural analyses of stage

14 egg chambers and isolated endochorions revealed that this layer contains three sublayers: the floor, the pillars and the roof (Margaritis et al., 1976; Petri et al., 1976; Margaritis et al, 1980). The inner endochorionic floor is flat and includes both solid and fenestrated regions separated from a thick outer roof layer by vertical pillars creating cavities that facilitate gas exchange. The roof constitutes a uniform layer showing ridges that define the bord. Finally, the exochorion consist of two layers of loose fibres which tend to be oriented parallel to the oocyte surface (Margaritis et al, 1980).

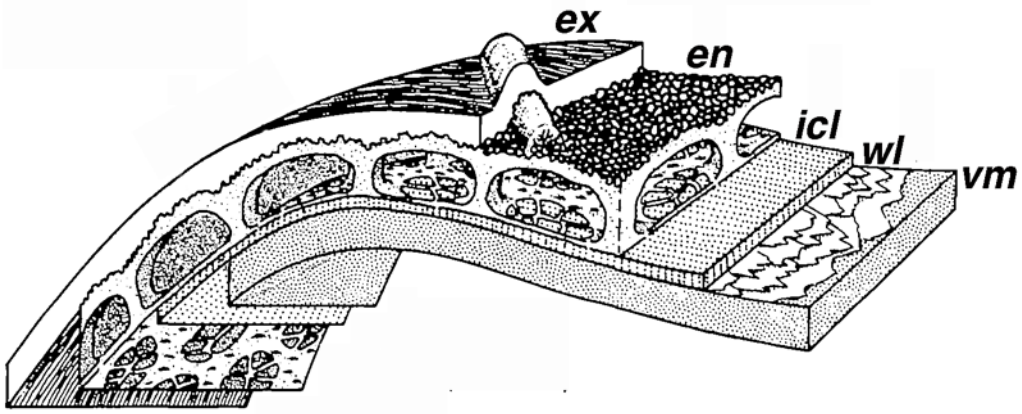

Figure.5 Three-dimensional representation of the eggshell. Three-dimensional representation of a fragment of the Drosophila eggshell main body indicating the relative orientation of its layers. Vitelline membrane (vm), wax layer (wl), innermost chorionic layer (icl), endochorion (en) and exochorion (ex) are indicated. (adapted from Margaritis et al., 1980).

1.5 Stabilisation of the eggshell

As described so far, the Drosophila eggshell is a highly specialised extracellular matrix formed over the course of approximately 30 hr during late oogenesis (Waring, 2000). The genes encoding major eggshell structural proteins are transcribed in follicle cells during stages 8–14 of oogenesis in a well-defined temporal order. The vitelline membrane genes are mainly expressed during mid-oogenesis at stages 8–10, while the chorion genes are transcribed from stage 11 onward (Waring, 2000; Cavaliere et al., 2008). Follicle cells produce and secrete eggshell proteins into the extracellular milieu,

where they assemble into a highly organized structure. Although the ultrastructure of the eggshell has been extensively studied little is known about the molecular mechanisms that underlie the assembly process.

In laid eggs, eggshell proteins are insoluble even in denaturing conditions due to the formation of covalent bonds among their proteins (cross linking). In the case of the chorion, it has been shown that cross linking among chorion proteins occurs in stage 14 and gives rise to the formation of di- and tri-tyrosine residues among eggshell proteins (Petri et al., 1976).Chorion cross linking is due to the Pxd peroxidase secreted by follicle cells in late oogenesis (Konstandi et al., 2005). Indeed, it has been shown that, at stage12-14, follicle cells secrete H_2O_2 and, accordingly, Pxd, in the presence of H_2O_2, is able to convert tyrosine residues to di-tyrosine ones. (Konstandi et al., 2005). Moreover, inhibition of peroxidase activity maintains chorion proteins soluble (Margaritis, 1985).

Conversely, in the case of the vitelline membrane, although it is not known whether a peroxidase is involved in its cross linking, it has been described the molecular mechanism that lead to its assembly. Major vitelline membrane components (sV23, sv17, VM32E and VM34C) (Fargnoli and Waring, 1982) are secreted between stage 9-10 and processed in a stage specific manner to favour protein-protein interaction among them (Pascucci et al., 1996). Indeed, all major vitelline membrane components contain a highly conserved hydrophobic domain (*a.k.a.* Vitelline Membrane or VM domain) of 38 amino acids enriched in cysteines and tyrosines that are necessary for protein-protein interaction (Andrenacci et al., 2001; Gigliotti et al., 1989; Scherer et al., 1988).

The hardening of the vitelline membrane occurs via two different cross linking events: the disulfide and the non-disulfide cross linking. The first one takes place at stage 14 and involves the formation of disulfide bridges among cysteine residues contained in the VM domain, as treatment with reducing reagents are sufficient to solubilise vitelline membrane proteins (Andrenacci et al., 2001; Manogaran and Waring, 2004; Wu et al., 2010). It has been shown that the deletions of the VM domain in vitelline membrane proteins, such as sV23 and VM32E, prevent their incorporation into the vitelline membrane and its assembly, leading to a collapse of the vitelline membrane as the egg is deposited (Andrenacci et al., 2001; Manogaran and Waring, 2004). However,

much less is known on the non-disulfide cross linking. As in the case of the chorion, it has been proposed that a still unidentified peroxidase would induce the formation of covalent bonds among vitelline membrane proteins (Cernilogar et al., 2001; LeMosy and Hashimoto, 2000).

Genetic mutations that affect the non-disulfide cross linking give rise to eggs that collapse soon after deposition or arrest very early in embryogenesis (Cernilogar et al., 2001; Degelmann et al., 1990; Degelmann et al., 1986; Ventura et al., 2010). The only genes identified so far to be important for the non-disulphide crosslinking are *fs(1)N, fs(1) Ph, closca* and *nudel* that will be extensively discussed in the following sections (see below).

Non-disulfide cross-linking occurs when the oocyte gets funnelled through the oviduct to be deposited (Heifetz et al., 2001). The passage through the oviduct swells the oocyte inducing an intracellular calcium rise that, in turn, triggers many events that are defined as "egg activation" (Heifetz et al., 2001; Kaneuchi et al., 2015). These events include: the resumption of the meiosis of the female pronucleus (Page and Orr-Weaver, 1997), the translation of maternal mRNAs (Tadros and Lipshitz, 2005), the degradation of maternal RNAs (Tadros et al., 2003), changes in the microtubular cytoskeleton (Theurkauf and Hawley, 1992) and, as mentioned, the non-disulphide vitelline membrane crosslinking (Horner and Wolfner, 2008). While in most animals the process of egg activation is linked with fertilisation, in *Drosophila melanogaster*, as in many insects, the two events are separated. While egg activation occurs in the passage through the oviduct, fertilisation occurs in the uterus when the activated oocytes pass close to the spermatheca (Heifetz et al., 2001).

Although the trigger for egg activation is not known, two models have been proposed. A first model proposed that the trigger consists in the passage of fluid enriched in Ca^{2+} from the walls of the oviduct into the oocyte (Heifetz et al., 2001). Alternatively, it has been suggested that the passage through the oviduct exerts mechanical pressure to the oocyte activating mechanosensitive ion channels that ultimately trigger egg activation (Horner and Wolfner, 2008). Since the two models are not mutually exclusive, it has been also proposed that swelling caused by the oviductal fluid would, in turn, induce mechanical stress to the oocyte causing the opening of the ion channels responsible for egg activation (Sartain and Wolfner, 2013). Therefore, the trigger for egg activation

and, thus, non-disulfide cross linking of the vitelline membrane remains poorly understood.

1.6 Embryogenesis

The egg is constituted for the first stages of embryogenesis by a syncytium (defined as syncytial blastoderm stage1-4). For the first seven divisions, nuclei, located in the central region of the egg, start to divide rapidly and synchronously until there are 128 nuclei in the central region of the egg. At this point, most of the nuclei, while continuing dividing, migrate towards the periphery surrounded by their local cytoplasmic domain (Karr and Alberts, 1986). After nine divisions, while most of the nuclei are close to the egg surface about 15 nuclei migrate from the egg centre towards the posterior pole. Soon these nuclei become separated into pole cells, the precursors of the germ line, and divide on at their own rate that differs from the rest of the embryo.

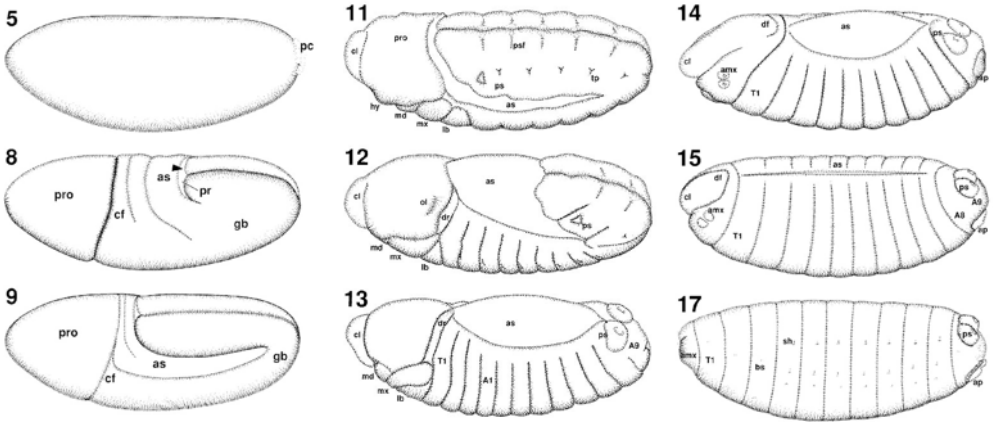

Figure.6 Overview of the main stages of embryogenesis (adapted from Hartenstein, 1993)

As the pole cells are forming, the remaining nuclei continue to divide for three more rounds of divisons in partially asynchronous manner. Indeed,these divisions occur in waves from the poles towards the middle of the embryo until the 13th division. As embryonic nuclei divide, they move out towards the egg surface (Foe and Alberts, 1983). During stage 5, the plasma membrane invaginates among the nuclei creating furrows between them that will eventually cut them into separate cells generating a celular blastoderm.

As cellularisation starts, mitotic divisions slow down. At this moment occurs the maternal to zygotic transition, meaning that while until this stage embryonic development has relied on mRNA maternally deposited in the oocyte during oogenesis, from this stage onwards, it starts to be under control of the zygotic genome (Bashirullah et al., 1999).

At stage 6 it starts gastrulation inducing complex morphogenetic movements that will give rise to the three germ layers and will shape the embryo internal organs. At the end of embryogenesis, morphogenesis will give rise to a segmented embryo containing: 3 head segments, 3 thoracic segments and 8 abdominal segments.

1.7 Establishment of embryonic axes

Specification of embryonic axes relies on positional information generated in the egg chamber. Using the segmented pattern of the larval epidermis, several genetic screenings allowed the identification of four different maternal systems that specify the embryonic axis.

In particular, the anteroposterior axis is specified by three different systems: the anterior, the posterior and the terminal system. On the other side, only one system, the dorso-ventral system, is responsible for the specification of the the dorso-ventral axis (Fig.7) (St Johnston and Nusslein-Volhard, 1992).

The anterior and posterior systems rely on the asymmetric localisation of mRNAs maternally deposited. Indeed, as described above, during oogenesis, the *bicoid* and *nanos* mRNAs are anchored at the anterior and posterior poles respectively of the egg and maintained quiescent. At the moment of egg activation, *bicoid* and *nanos* mRNAs are translated forming a gradient of Bicoid anteriorly and a gradient of Nanos posteriorly. These two proteins act as morphogenes, acting as concentration dependent activators for a series of zygotic genes known as “gap genes”, as their mutation cause deletion of large portions of the larval cuticle (Struhl, 1989).

In contrast to the anterior and posterior systems, in the terminal and dorso-ventral systems, the central element of the pathway is a transmembrane receptor uniformly localised at the embryo surface (Anderson et al., 1985b; Casanova and Struhl, 1989). Moreover, in both systems, one gene is locally expressed by follicle cells in the egg

chamber (Furriols et al., 2007; Stein and Stevens, 1991; Stevens et al., 1990) rather than in the germline, and lead to the deposition of a asymmetric signal in the vitelline membrane (Zhang et al., 2009a). Hence, in embryogenesis, asymmetric cues in the vitelline membrane trigger the restricted activation of the terminal and dorsoventral receptors, Torso (Tor) and Toll (Tl) respectively, in restricted domains. In particular, activation of the Tor receptor at the embryonic poles activates the transcription of the terminal gap genes *tailless* and *huckebein* (Klingler et al., 1988; Strecker et al., 1989; Weigel et al., 1990). Activation of Tl at the embryonic ventral side allows the translocation of the transcription factor Dorsal to the nucleus. Graded activity of Tl giving rise to a gradient of nuclear concentration of Dorsal along the embryonic DV axis driving the transcription of different zygotic genes (Roth et al., 1989).

Therefore, positional information from the four maternal systems determines the activation of the zygotic genes in spatially distinct domains. In the antero-posterior axis, gap genes encode for transcription factors that, in turn, trigger the differential activation of another set of genes named "pair rule" as their mutations affect every alternate segment eliminating a part of the cuticle pattern (Nusslein-Volhard and Wieschaus, 1980). Finally, pair rule genes activate the segment polarity genes that, in turn, activates homeotic genes to define the identity of each segment (Fig.8).

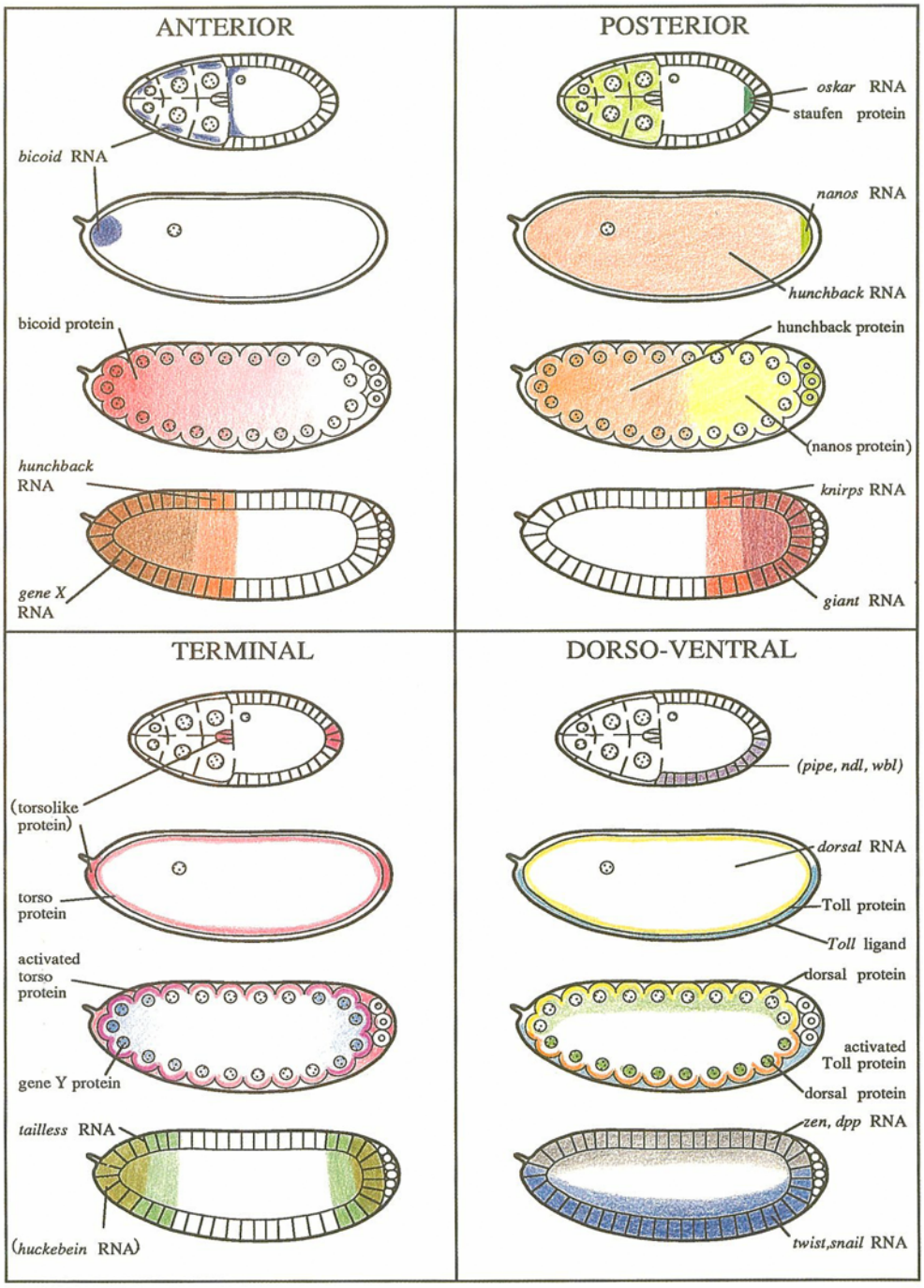

Figure.7 Overview of the four maternal system that establish the embryonic axes (adapted from St Johnston and Nusslein-Volhard, 1992).

Therefore, the inputs from the four maternal system is converted into narrow domains of expression of zygotic genes that define embryonic identity (Lawrence, 1992).

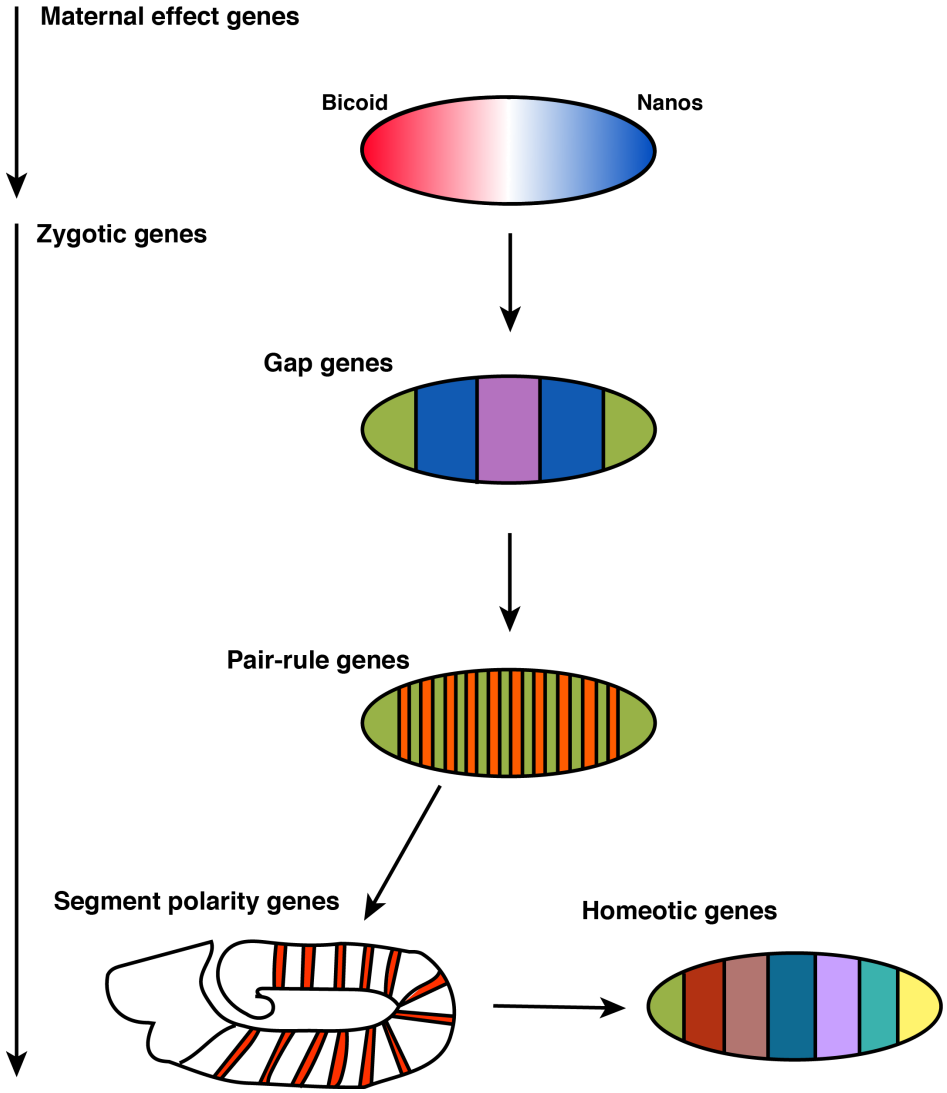

Figure.8 Antero-posterior axis specification. Maternal effect genes as *bicoid* and *nanos* lead to the activation of gap genes that trigger the differential activation of another set of genes named pair rule. Pair rule genes activate the segment polarity genes that, in turn, activate homeotic genes and specify the identity of each segment (adapted Gilbert, 1988).

1.8 The terminal system

The terminal system is responsible for the specification of the most anterior and posterior embryonic structures. While terminal signalling is sufficient for the development of posterior terminal structures, anterior terminal structures need the cooperation between terminal and anterior system (Frohnhofer et al., 1986;Bellaiche et al., 1996). Genetic screens for maternal effect mutations that affect embryonic

patterning led to the identification of an entire class of genes named as the “terminal” group.

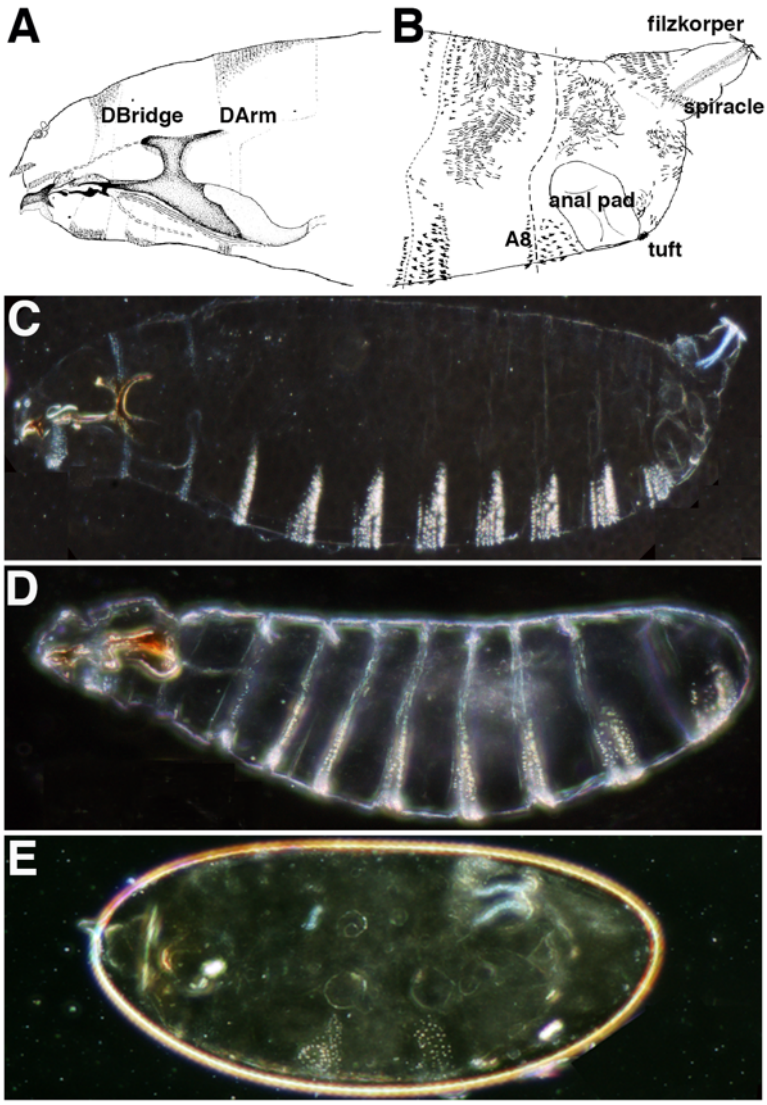

Figure.9 Cuticular structures specified by the terminal system (A-B) Anterior and posterior cuticular structures specified by the terminal system. **(A)** Dorsal arm and dorsal bridge. **(B)** Denticle belts of the 7th (not shown) and 8th abdominal segment, the spiracles and the filzkörper, the Anal Sense Organ (not shown), the tuft and the anal pads (adapted from Jurgens, G. and Hartenstein, V., 1993). (**C**) cuticle of a wild-type embryo. (**D**) cuticle of embryos displaying a terminal phenotype displaying loss of terminal structure. (**E**) cuticle of an embryo displaying the “spliced” phenotype where terminal structures expand at the expenses of the others. Note the loss of lots of abdominal denticle belts and an ectopic filzkörper in the middle of the embryo.

At the cuticular level mutations in the genes of the "terminal group" cause the lack of the labrum and reduced chitin mandibular structures such as the dorsal arm, the ventral arm and the dorsal bridge (Fig.9A and 9D) (Schupbach and Wieschaus, 1986). At the posterior pole loss of terminal activity cause lack of all structures posterior to the the 7^{th} abdominal segment such as: denticle belts of the 7^{th} and 8^{th} abdominal segment, the spiracles and the filzkorper, the Anal Sense Organ, the tuft and the anal pads (Fig.9B and 9D) (Schupbach and Wieschaus, 1986; Jurgens, G. and Hartenstein, V., 1993).
Furthermore, loss of terminal signalling posteriorly leads to cellularisation defects also known as the "pole–hole" phenotype (Degelmann et al., 1986; Schupbach and Wieschaus, 1986). This phenotype is not due to apoptosis but to a change in cell fate caused by the influence of the pole cells (de Las Heras et al., 2009).
Conversely, hyperactivation of terminal signalling cause the "spliced" phenotype where terminal embryonic structures expand at the expenses of thoracic and abdominal structures (Fig.9E) (Schupbach and Wieschaus, 1989; Strecker et al., 1989). Genetic analysis demonstrated that mutations responsible for the spliced phenotype consisted in gain of function mutation in the *torso* (*tor*) gene (Strecker et al., 1989;Sprenger and Nusslein-Volhard, 1992; Casanova and Struhl, 1993). These findings put *tor* as the central element of the terminal system and suggested that, although uniformly localised in the plasma membrane, Tor was activated at the poles (Casanova and Struhl, 1993).

1.9 Torso, the central element of the terminal system

The *tor* gene encodes for trans-membrane receptor tyrosine kinase (RTK) that contains an N-terminal signal peptide, a trans-membrane domain, and a C-terminal region that show significant homology to the tyrosine kinase domains of other receptors (Sprenger et al., 1993). Although *tor* mRNA is synthesized during oogenesis by nurse cells (Sprenger and Nusslein-Volhard, 1992), the protein is not translated until egg activation, when it localises uniformly at the embryonic plasma membrane (Casanova and Struhl, 1989). The identification of Tor as a RTK and the sequencing of its alleles allowed to have a molecular explanation to the phenotypes of loss and gain of function identified so far. Sequencing of *tor* null mutants, showed that all these mutations were affecting the intracellular domain of Tor involved in initiating the intracellular

signaling cascade (Sprenger et al., 1993). Conversely, sequencing of point mutations that give rise to a gain of functions alleles were shown to be affecting the extracellular domain of Tor involved in ligand recognition allowing ligand independent Tor dimerisation (Sprenger et al., 1993; Casanova and Struhl, 1993). The employment of *tor* gain of function alleles allowed identifying the genes that were downstream and upstream of *tor*. Therefore, it was found that mutations in *corkscrew, Draf,* and *Dsor1*, were able to suppress the *tor* gain of function phenotype suggesting that these genes were encoding for the transducers of Torso signalling (Perkins and Howells, 1992; Casanova and Struhl, 1993;). On the other hand, mutations in *torso-like(tsl), trunk(trk), fs(1)polehole (fs(1)ph), fs(1)Nasrat (fs(1)N)* and *closca* (*clos*) did not affect the Tor gain of function phenotype indicating that these genes were involved in the production of the Tor ligand (Stevens et al., 1990; Casanova and Struhl, 1993; Ventura et al., 2010). In the next paragraphs we will describe the genes upstream of Tor and their possible function in generating the Tor ligand.

1.10 Torso-like, the key element of the terminal system

The *torso-like* (*tsl*) gene is the only one belonging to the terminal group, that is not expressed in the germ line. Mosaic analysis showed that *tsl* is expressed by specific subpopulations of follicle cells in the egg chamber located at the anterior and posterior poles of the oocyte (Stevens et al., 1990). In particular, border cells and centripetal express *tsl* at the anterior pole while posterior follicle cells express it at the posterior (Stevens et al., 1990; Furriols et al., 2007). *tsl* mutant flies lay embryos that show lack of terminal structures and, conversely, flies where *tsl* has been ectopically expressed in all follicle cells, give rise to embryos displaying the spliced phenotype, indicating an ectopic activation of Tor (Savant-Bhonsale and Montell, 1993). This observation together with the finding that the Tsl protein was localised at the embryo poles was taken as an indication that *tsl* encoded the Tor ligand (Martin et al., 1994). However, later studies suggested, alternatively, that the *trk* gene encoded for the Tor ligand (Casanova et al., 1995; Casali and Casanova, 2001). Moreover, Tsl accumulation at the plasma membrane could not be reproduced (Stevens et al., 2003). Conversely, it was demonstrated that Tsl accumulates at the poles at the inner surface of the embryonic vitelline membrane indicating that restricted Tsl localisation was, somehow, involved

in restricting Tor activation (Stevens et al., 2003). Finally, identification within the Tsl sequence of a domain homologous to the Membrane Attack Complex Perforin (MACPF) domain (Pointing, 1999) further strengthened the hypothesis that Tsl was unlikely to act as the Tor ligand. Indeed, proteins containing the MACPF domain are involved in the formation of pores as defense mechanism in the vertebrate innate immune system (Fig.11) (Ponting, 1999). Since this domain is crucial to have hints about the function of Tsl, we will briefly describe the role of MACPF proteins in the innate immune response.

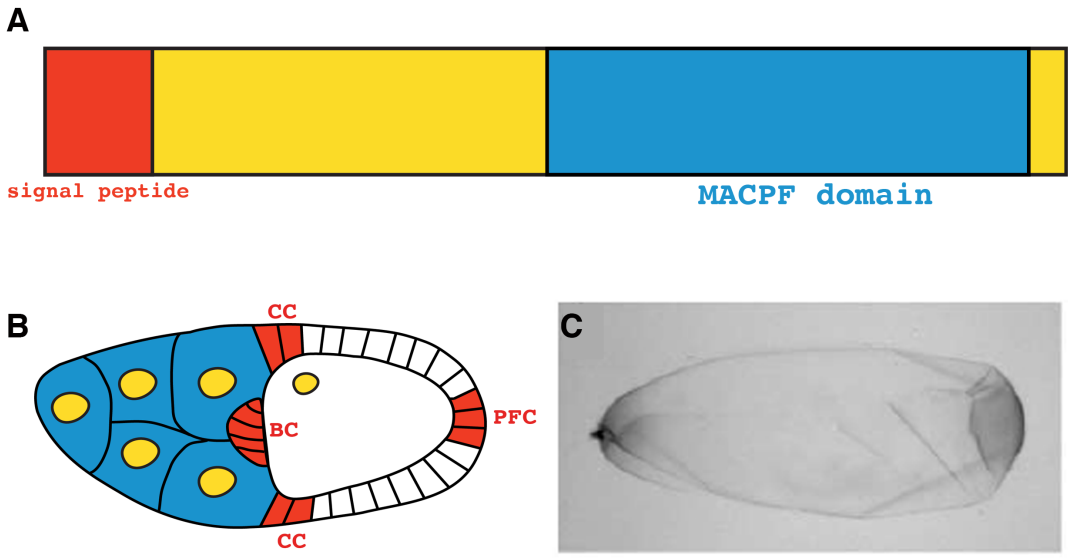

Figure.10 Torso-like expression and protein localisation (A) Schematic diagram of the Tsl protein containing an amino-terminal signal peptide and the MACPF domain close to the carboxy-terminus. **(B)** Schematic diagram of follicle cells populations expressing the *tsl* gene according to Martin et.al, 1994. Posterior follicle cells (PFC), border cells (BC) and Centripetal cells (CC). **(C)** Empty vitelline membranes from early embryos stained with an anti-Tsl antibody showing the accumulation of Tsl in the inner surface of the vitelline membrane (adapted from Stevens et al., 2003).

1.11 MACPF proteins in the immune response

The membrane attack complex/perforin (MACPF) domain was identified and named as being common to five complement proteins (C6, C7, of C8α, C8β,and C9) and

perforin (Tschopp et al., 1986). These proteins perform critical functions in innate and adaptive immunity.

The complement system consists of a number of small proteins found in the blood, and circulating as inactive precursors. Upon recognition of a Gram-negative pathogen, complement factors C6 to C9 assemble to form a scaffold (the membrane attack complex (MAC)) that permits C9 polymerization into pores that lyse Gram-negative pathogens.Indeed, the C6 and C7 proteins attach to the plasma membrane surface of the target cell and allow the insertion of C8α into the phospholipid layer through a trans-membrane α-helix firmly anchoring the complex to the membrane and recruiting C9 (Rosado et al., 2007). Between 12 and 18 C9 molecules assemble to form a complete ring that undergoes conformational change to form a doughnut-shaped pore that is postulated to kill the cell through osmotic lysis (Biesecker et al., 1993; Kondos et al., 2010).

While complememement proteins freely circulate in the plasma, perforin is compartmentalized in acidified secretory granules in cytotoxic T lymphocytes (CTLs) and natural killer cells (Voskoboinik et al., 2006). Upon recognition of cells that have been infected by a virus CTLs or Natural Killer cells that attach closely to the target cell forming the "immune synapse" and releasing secretory granules containing perforin. The extracellular environment is highly enriched in Ca^{2+} that binds to a specific domain in the perforin protein allowing its interaction with the plasma membrane of the target cell (Voskoboinik et al., 2005). Membrane bound Perforin forms a pore that spans the plasma layer through its amphipathic β-strands (Law et al., 2010). In this way, pro-apoptotic serine proteases (called granzymes), secreted by lymphocytes together with Perforin, are uptaken by the target cells through the pores formed by Perforin aggregates and trigger the apoptosis of the infected cell. Alternatively, it has been suggested that the permeabilisation of the plasma membrane by Perforin is followed by a "wound healing response" that would trigger an endocytosis-like event in addition to pore formation guaranteeing the delivery of granzymes into target cells (Fig.11) (Keefe et al., 2005; Praper et al., 2011).

In addition to proteins involved in immune defense, nonlytic proteins, such as Astrotactin, which is involved in glia-guided axon pathfinding, have a MACPF domain (Zheng et al., 1996;Ponting, 1999).Therefore, it has been proposed that the MACPF

fold might have been used in evolution for nonlytic purposes, such as conformational flexibility and membrane insertion (Rosado et al., 2007) . Unfortunately, even among MACPF proteins the degree of sequence similarity is extremely low, and as the structure of very few MACPF proteins has been charachterised, the structural similarity of Tsl to any other MACPF protein is also unknown (Voskoboinik et al., 2006). Therefore, it is not possible to predict whether Tsl is able to form a pore or whether the MACPF domain might have a different function in the context of the the terminal system.

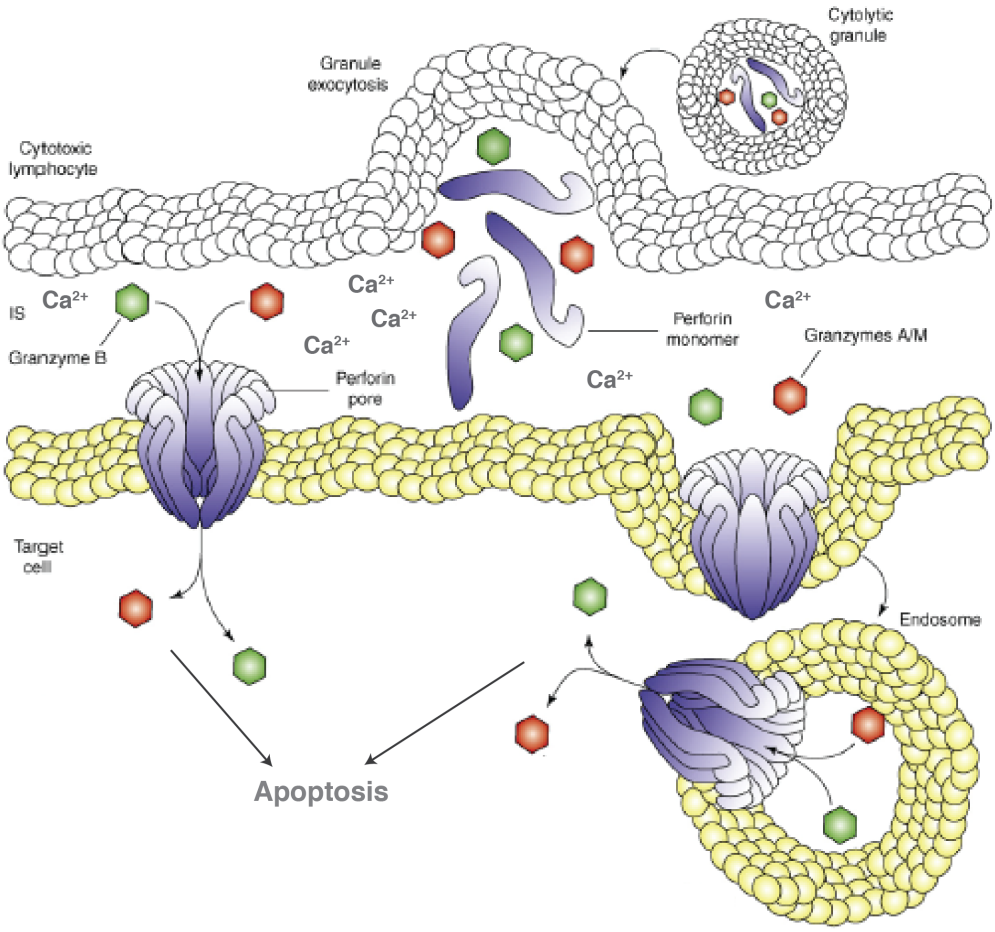

Figure.11 Delivery of Perforin and granzymes by cytotoxic lymphocytes. Contents of cytolytic granules such as perforin and granzymes are released into the extracellular space upon recognition of a target cells. As the extracellular space is encriched in $Ca^{2+,}$ perforin binds to the target cells plasma membrane allowing the entry (either via formation of a pore either through endocytosis) of granzymes that trigger apoptosis of the the target cell (adapted from Bolitho et al., 2007).

1.2 Trunk

The *trk* gene was identified as giving rise to a terminal phenotype as *tor* or *tsl* mutants (Schupbach and Wieschaus, 1986). *trk* encodes for a secreted protein of 226 aminoacids expressed as in the germ line by nurse cells during oogenesis and accumulated in the oocyte as mRNA until it is translated in early embryogenesis (Fig.12B) (Casanova et al., 1995). The C-terminal domain of Trk has an arrangement of cysteines similar to the cystine knot motif found in many growth factors and ligands (Fig.12A) (Casanova et al., 1995; McDonald and Hendrickson, 1993; Murray-Rust et al., 1993) . While the features of Trk suggested that it might be the Tor ligand, Trk is uniformly distributed in the oocyte and, thus, seemed unlikely to activate Tor at the poles. However, the observation that Trk displays putative cleavage sites suggested that Trk was proteolitically cleaved and, accordingly, a single amino acid change at a putative cleavage site abolishes Trk function (Casanova et al., 1995). Later studies showed that a C-terminal fragment of the Trk protein can activate the Torso receptor pathway even in *fs(1)N*^{12} and *fs(1)ph*^{1901} or *tsl* mutant backgrounds (Casali and Casanova, 2001). On the other hand, ectopic expression of *tsl* still requires *fs(1)N*^{12} and *fs(1)ph*^{1901} and *trk* function to give rise to the spliced phenotype associated with ubiquitous Tor activation (Casanova et al., 1995; Furriols et al., 1998; Stevens et al., 2003). Altogether, these observations suggest that *trk* is downstream of *tsl* and envisaged a scenario where localised Tor activation relies on the restricted proteolysis of Trk probably upon the action of Tsl (Casali and Casanova, 2001; Furriols and Casanova, 2003). Later studies showed that Trk is cleaved *in vivo* and that this processing is important for its function, as a single amino acid substitution in a cleavage site of the Trk protein abolishes its cleavage and acts as a null mutation (Fig.12C) (Henstridge et al., 2014; Casanova et al., 1995)). Moreover, it was found that the Trk is cleaved independently of Tsl by the redundant activity of pro-protein convertases as Furin1 and Furin 2 involved in the intracellular maturation of many pro-hormones (Henstridge et al., 2014; Johnson et al., 2015). The observation that Trk is likely to be cleaved intracellularly argued against the role of Tsl in controlling Trk restricted activation (Johnson et al., 2015). suggested that Tsl might be involved in the localised secretion of an active cleaved form of Trk at the poles (Duncan et al., 2014; Johnson et al., 2015). Therefore, studies on Trk have provided two different models for

restricted Tor activation. The two models will be described in details in the following sections.

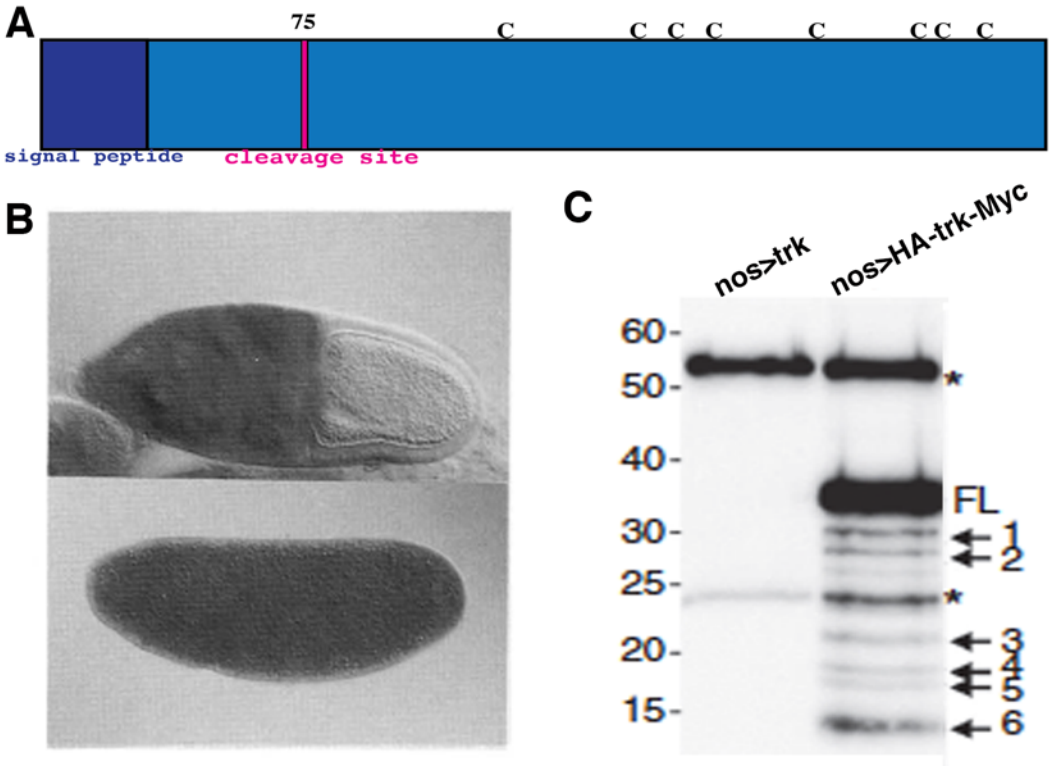

Figure.12 Trunk gene expression and cleavage A) Schematic diagram of the Trk protein showing an amino-terminal signal peptide, a cleavage site at position 75 and seven cysteines (indicated as C) forming the cysteine knot motif. (adapted from Casanova et al., 1995). **B**) *In situ* hybridisation with a *trk* mRNA probe showing its expression in nurse cells in the egg chamber (top) and its accumulation in the early embryo (bottom) (adapted from Casanova et al., 1995). **C**) Immunoblot of HA-Trk-Myc using an anti-Myc antibody. Immunoblot of protein extracts immunoprecipitated (using anti-Myc) from embryos overexpressing HA-Trk-Myc in the germ line and blotted with anti-Myc. HA:Trk:Myc extracts contain the full-length (FL) Trk protein and six C-terminal Trk fragments (arrows) not observed in untagged Trk extracts (nos>Trk). Asterisks indicate non-specific bands (adapted from Henstridge et al., 2014).

1.13 The *fs(1)Nasrat*, *fs(1)polehole* and *closca* genes

The *fs(1)Nasrat (fs(1)N), fs(1)polehole (fs(1)ph)* and *closca(clos)* genes were identified as being important for Tor signaling as specific mutations gave rise to lack of terminal strucutures (Degelmann et al., 1986, Ventura et al., 2010)). *fs(1)N), fs(1)ph* and *clos* play a role in the integrity of the eggshell as females bearing null mutations in any of

these genes lay eggs that collapse after egg laying and that fail to sustain embryonic development (Degelmann et al., 1986; Degelmann et al., 1990; Cernilogar et al., 2001;Ventura et al., 2010). This phenotype is due to a failure in the non-disulfide cross-linking of the vitelline membrane (Cernilogar et al., 2001; Jimenez et al., 2002; Ventura et al., 2010). However, specific point mutations have been identified, *fs(1)N*^{12}, *fs(1)ph*^{1901} and *clos*^{1}, that do not give rise to eggs that collapse. Indeed, females bearing any of these mutations lay eggs that are able to sustain embryonic development but lack terminal structures (Degelmann et al., 1990; Jimenez et al., 2002; Ventura et al., 2010)).

fs(1)N, fs(1)ph and *clos* encode for proteins that have a signal peptide, are rich in leucine residues (12%) and have many N-linked glycosylation motifs. In particular, the *fs(1)N* gene encodes for the Nasrat protein containing 2118 aminoacids (Jimenez et al., 2002). Similarly to *fs(1)N, fs(1)ph* and *clos* encode for the Polehole and Closca proteins respectively, containing around 1800 aminoacids and that share a significant sequence similarity between them and, although to a lesser extent, with Nasrat. (Jimenez et al., 2002; Ventura et al., 2010). Unfortunately, besides the similarities among them, Nasrat, Polehole and Closca do not show any similarity with other proteins making difficult to assign a specific molecular function to any of them.

At stage 9 of oogenesis, the three proteins are secreted by the oocyte and accumulate in the nascent vitelline membrane (Ventura et al., 2010). Nasrat, Polehole and Closca are reciprocally required for their extracellular accumulation meaning that, in egg chambers from flies bearing a null mutation for any of them, the other two proteins do not accumulate extracellularly (Jimenez et al., 2002; Ventura et al., 2010). This observation suggested that the three proteins might be forming a complex at the vitelline membrane although it has not been possible to detect physical interaction among them (Jimenez et al., 2002).

Nasrat and Polehole were considered proteins with multiple domains: one for terminal function and the other for vitelline membrane integrity (Degelmann et al., 1990). Hence, the *fs(1)N*^{12} and *fs(1)ph*^{1901} point mutations were thought to specifically impair the terminal function of Nasrat and Polehole without affecting the function of these proteins in vitelline membrane cross-linking (Cernilogar et al., 2001). Morever, the presence of independent functional domains was corroborated by genetic studies

describing a particular *fs(1)N* allele, the the *fs(1)N*^{4} allele (Degelmann et al., 1990). In particular, all eggs from homozygous *fs(1)N*^{4} females collapse due to defects in vitelline membrane non-disulphide cross linking (Cernilogar et al., 2001). The same phenotype is observed in eggs from hemyzigous *fs(1)N*^{4} females and in the ones from transheterozygous females of *fs(1)N*^{4} over a null *fs(1)N* allele (Degelmann et al., 1990). All these observations suggested that this particular allele of the *fs(1)N* gene behaves as a null. However, eggs from transheterozygous females of *fs(1)N*^{4} over the *fs(1)N*^{12} terminal allele give rise to *wild-type* larvae and adults indicating an intragenic complementation between the two alleles (Degelmann et al., 1990). These data indicated that the Nasrat protein had two distinct domains: one for vitelline membrane integrity (which would be disrupted in the *fs(1)N*^{4} mutant) and another for terminal patterning (disrupted in the *fs(1)N*^{12} mutant). However, in the case of *clos*, the point mutation *clos*^{1} give rise to embryo that display lack of terminal structures but also with an impaired vitelline membrane cross-linking (Ventura et al., 2010). Moreover, eggs laid by *clos*^{1} females are soft and permeable to the neutral red dye indicating defects in vitelline membrane integrity. These data might suggest lack of clear independent domains in Closca or, alternatively, that the *clos*^{1} mutation is affecting a region of the protein required for both domains (Ventura et al., 2010).

The role of Nasrat, Polehole and Closca in terminal patterning has been related to their function in anchoring or stabilising the Tsl protein in the egg chamber. Indeed, Tsl extracellular accumulation in the egg chamber requires Nasrat, Polehole and Closca as extracellular Tsl is not detected in egg chambers from *fs(1)N, fs(1)ph* or *clos* null mutant mothers (Jimenez et al., 2002; Ventura et al., 2010)). Accordingly, strongly reduced Tsl levels were detected in the inner side of the vitelline membrane of embryos laid by *fs(1)N*^{12} and *fs(1)ph*^{1901} mothers corroborating the role of Nasrat and Polehole in stabilising or anchoring the Tsl protein at the vitelline membrane (Stevens et al., 2003). Nevertheless, upon ectopic expression in all follicle cells in egg chambers from *fs(1)N*^{12} and *fs(1)ph*^{1901} mothers, high Tsl levels can be detected at the vitelline membrane even though they do not rescue the lack of terminal structures (Stevens et al., 2003). These data suggested that Nasrat, Polehole and Closca had an additional role in terminal patterning besides stabilising/localising Tsl and it was proposed that they are required for "Tsl to exert its function" (Stevens et al., 2003; LeMosy, 2003).

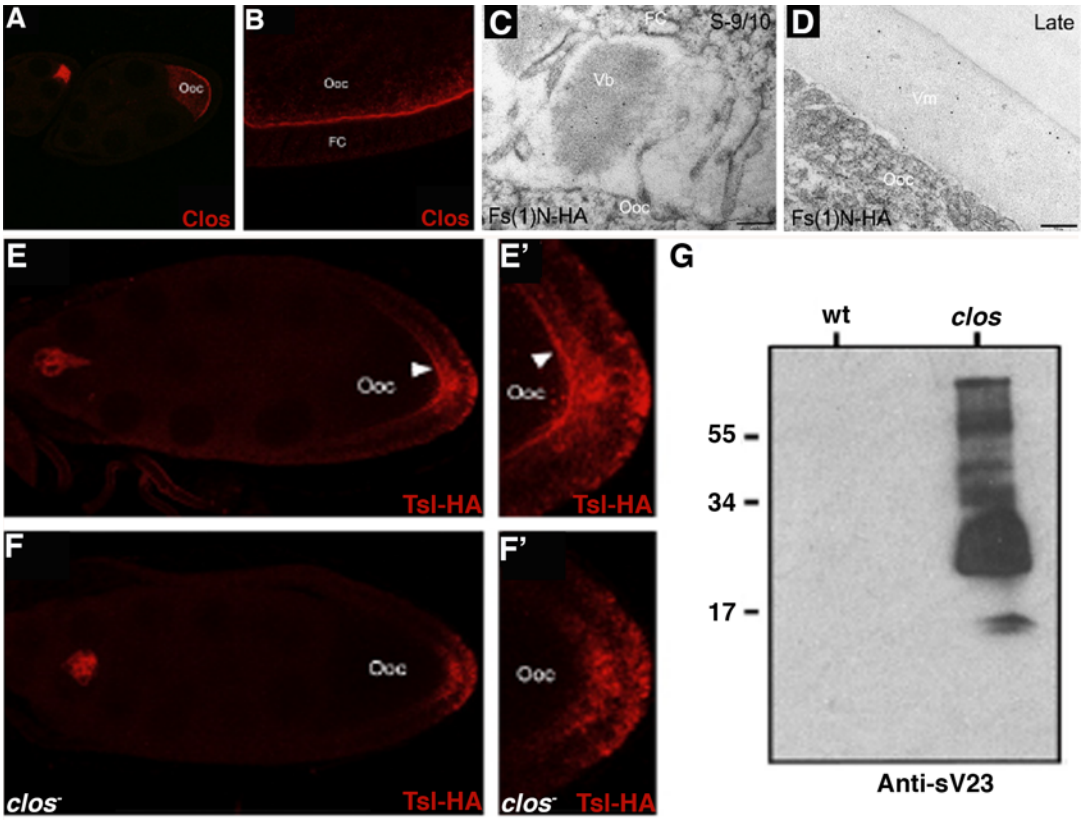

Figure.13 The Nasrat, Polehole and Closca proteins (A) Stage 7/8 (left) and stage 9 (right) egg chambers stained with the anti-Clos antibody. Clos is detected at early stages inside the oocyte and by stage 9 accumulates at the oocyte surface. **(B)** Magnification of a stage 10 egg chamber stained with the anti-Clos antibody. **(C-D)** Immunoelectron microscopy of egg chambers from Nasrat-HA flies stained with HA. C) At stage 9, Nasrat localises in the vitelline bodies (Vb). **(D)** In late egg chambers Nasrat is detected in the vitelline membrane. Vm (Vitelline mebrane), Ooc (Oocyte), FC (follicle cells). **(E)** Stage 9 egg chamber from Tsl-HA females stained with anti-HA antibody. **E′)** Magnification of the same egg chamber shown in E. F) Stage 9 *clos* null egg chamber from Tsl-HA females stained with anti-HA antibody. **(F′)** Magnification of the same egg chamber shown in F. Note that Tsl-HA does not accumulate extracellularly in these egg Ooc, oocyte. **(G)** Western blot analysis with an antibody against the vitelline membrane protein sV23 of protein extracts from embryos laid by wild-type and *clos* null mutant females. As *clos* is required for non-disulphide vitelline membrane crosslinking, soluble sV23 protein can be detected in embryonic extracts from clos null mutant flies. All images were adapted from Ventura et al., 2010.

1.15 Models for Tor restricted activation

As mentioned above, localisation of Tsl at the poles both in the egg chamber and at the embryo plasma membrane was taken as an indication for it being the Tor ligand (Savant-Bhonsale and Montell, 1993; Martin et al., 1994)). Therefore, once secreted by follicle cells, Tsl would remain tethered to the oocyte surface or to the vitelline membrane during oogenesis and activate Torso receptor in early embryogenesis (Degelmann et al., 1990; Stevens et al., 1990; Stevens et al., 2003). In this scenario, restricted terminal activity would rely on the delayed delivery of a localised ligand (Furriols and Casanova, 2003).

However, the identification of the *trk* gene and its cloning strongly suggested that it encodes for the Tor ligand ((Schupbach and Wieschaus, 1986; Casanova et al., 1995). Although, uniformly distributed in the early embryo, it was proposed that, as Trk presented many cleavage sites along its sequence, restricted Tor activation might rely on the localised processing of Trk (Casanova et al., 1995). This idea was further strengthened by the observation that C-terminal fragments of the Trk protein can activate the Torso pathway even in embryos laid by *fs(1)N*^{12}*, fs(1)Ph*^{1901} or *tsl* females (Casali and Casanova, 2001). In particular, analysis of the *trk* sequence unveiled the existence of two putative cleavage sites and, accordingly, two different deletions generated at each site that retained 159 and 108 amino acids respectively of the carboxy-terminus of the protein (named Trk$^{C\text{-}159}$ and Trk$^{C\text{-}108}$) were both able to activate Tor signaling in the embryos upon injection of their mRNA and upon misexpression in the germ line (Casali and Casanova, 2001).

These data suggested a model where restricted Tor activation at the embryo poles would rely on the local processing of a uniform signal (Furriols and Casanova, 2003). Moreover, as the Trk$^{C\text{-}159}$ retains the ability to bypass the requirement for Tsl, it was speculated that Trk proteolysis occurred in two steps: a first Tsl-dependent cleavage would generate the Trk$^{C\text{-}159}$ and a second Tsl-independent one would give rise to the Trk$^{C\text{-}108}$ that, probably, mimics the active form of the ligand (Casali and Casanova, 2001). In this scenario, the role of Tsl would be to nucleate at the poles a protease that triggers restricted proteolysis of Trk (Furriols and Casanova, 2003; Li, 2005). Accordingly, Perforins are secreted by lymphocytes together with proteases (granzymes), so it might be that the Trk processing protease is anchored at the vitelline

membrane together with Tsl or, alternatively, that the activity of a uniformly distributed protease might be regulated by Tsl at the poles (Fig.14).

However, this model was recently challenged by new data that suggested another scenario for Tor activation at the poles (Duncan et al., 2014). Indeed, Trk is cleaved *in vivo* multiple times generating multiple carboxy-terminus fragments and this is likely to be due to the activity of the Furin1 and Furin2 pro-protein convertases (Henstridge et al., 2014; Johnson et al., 2015). Nevertheless, it was found that Trk cleavage pattern was not altered in embryos laid by *tsl* mutant mothers suggesting that Tsl was unlikely to be involved in Trk proteolysis (Henstridge et al., 2014). Moreover, it was found that an amino-terminus fragment of the Trk protein (N:Trk) needed the Tsl protein for its extracellular accumulation (Johnson et al., 2015).

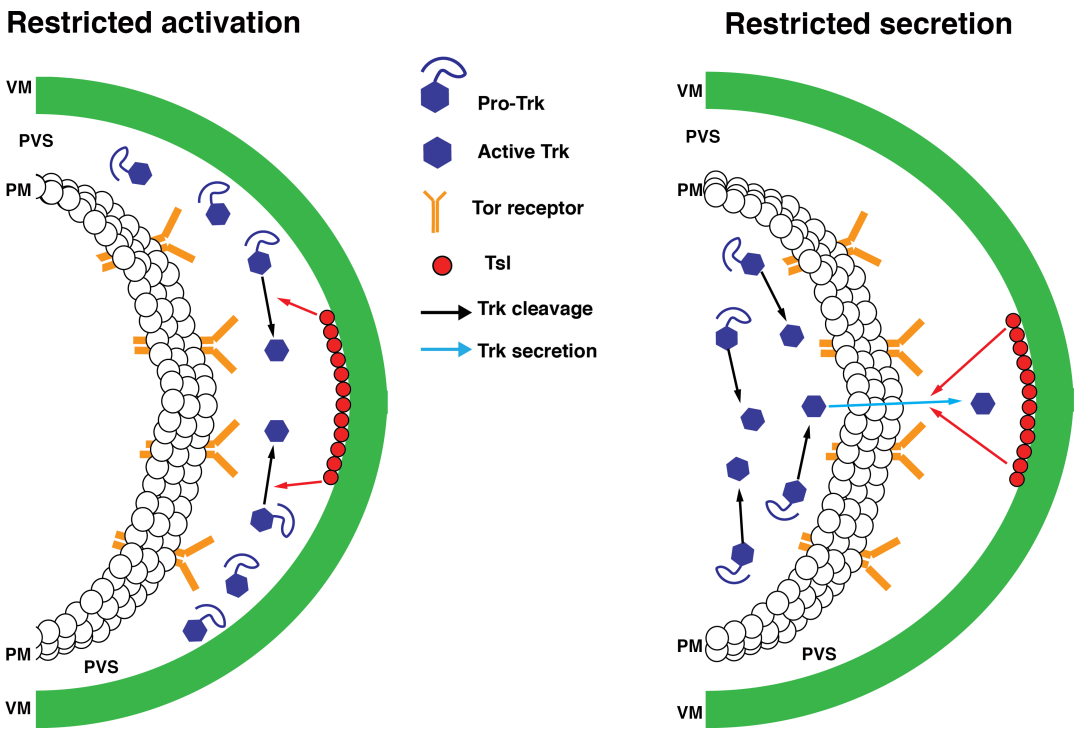

Figure.14 Models for Tor restricted activation. Schematic diagram for Tor restricted activation at the poles of the embryonic syncytial blastoderm. In the restricted activation model (left), Trunk is secreted into the perivitelline space (PVS), the space between the vitelline membrane and the embryo plasma membrane, where it is activated upon cleavage mediated by Tsl at the poles of inner surface of the vitelline membrane. In this way, Trk cleavage only occurs at the poles and allows Trunk to activate Tor (adapted from Casali and Casanova, 2001and Furriols and Casanova, 2003). In the "restricted secretion" model (right), Trk activation occurs upon cleavage within the embryo prior to Trk secretion. Vitelline

membrane tethered Tsl interacts with the embryo plasma membrane to regulate the restricted secretion of the active cleaved Trk from the embryo poles to activate Tor (adapted from Duncan et al., 2014). PM (Plasma membrane), PVS (PeriVitelline Space), VM (Vitelline Membrane).

These finding led to a model where Trk cleavage occurs intracellularly by the Furin1 and Furin2 pro-protein convertases and Tsl would mediate the extracellular accumulation of the cleaved active form of Trk at the embryo (Duncan et al., 2014; Johnson et al., 2015)). Indeed, MACPF proteins that can induce the membrane repair response and trigger defense related secretory molecules (Reddy et al., 2001). In this scenario, membrane damage induced by Tsl might induce the exocytosis of the active cleaved form of Trk from the embryo poles via the membrane repair mechanism or via the formation of a pore (Johnson et al., 2015) (Fig.14).

1.16 Downstream of Torso

Upon ligand binding, Torso, as all the RTKs, dimerises and undergoes autophosphorylation on several tyrosine residues (Sprenger and Nusslein-Volhard, 1992). Phosphorylation of Tor triggers the Ras/Raf/MAPK pathway to regulate the expression of the target gap genes: *tailless* (*tll*) and *huckebein* (*hkb*) (Strecker et al., 1989; Weigel et al., 1990).

Phosphoryation of Tor at the poles creates a "gradient" of MAPK that correlates with differential gene expression, as low levels of Tor activity are sufficient to activate *tll* but not *hkb* (Furriols et al., 1996) . Rather than actively promoting the expression of *tll* and *hkb*, the Tor signaling antagonises, at the poles, a ubiquitous repressor present throughout the whole embryo encoded by the *capicua* (*cic*) gene (Jimenez et al., 2000). Mutations in *cic* lead to expansion of the expression of *tll* and *hkb* and to a cuticular phenotype similar to the spliced phenotype observed with Tor gain of function alleles (Jimenez et al., 2000). Cic encodes for a protein that has a DNA binding domain of the HMG box class and interacts with Groucho (Gro) a transcriptional co-repressor found in many developmental contexts (Jimenez et al., 2000; Fores et al., 2015)).,As for *cic*, mutations in *gro* give rise to an expansion of *tll* and *hkb* (Paroush et al., 1997).

Although *cic* mRNA is uniformly distributed in the embryo, the Cic protein localises in the nuclei of the central region of the embryo but it is excluded at the poles by Tor

signaling (Jimenez et al., 2000). Altogether, these data suggested that Cic could act as a DNA-binding protein recruiting Gro to the *tll* and *hkb* promoters acting as a transcriptional repressor (Furriols and Casanova, 2003). In this way, Cic would be the link between Torso signaling and transcriptional gene regulation (Jimenez et al., 2000). In support of this idea, it has been suggested that upon Tor signalling the Cic protein is phosphorylated and shuttled from the nucleus to the cytoplasm where it is sent to degradation (Astigarraga et al., 2007;Grimm et al., 2012).

Therefore, Tor activation generates a graded MAPK activity at the poles that oppose a complementary gradient of Cic that decreases towards the pole (Jimenez et al., 2012).

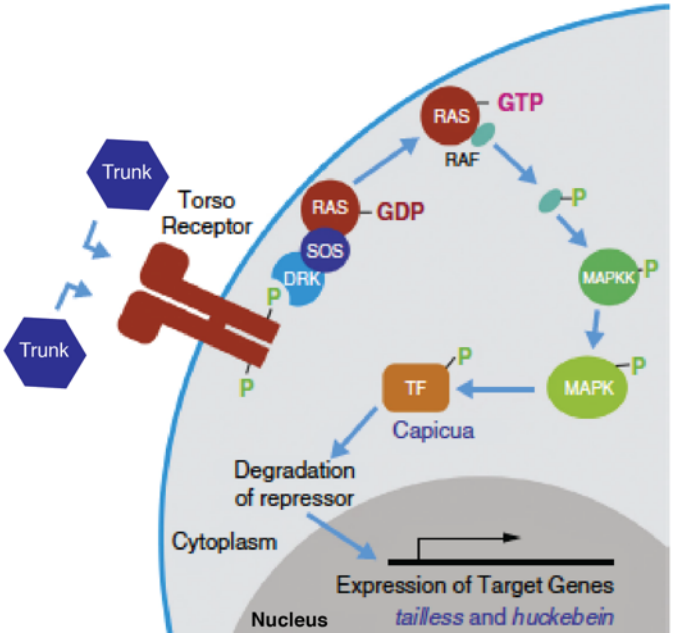

Figure.15 Molecular mechanisms of Torso signalling. Intracellular signalling from an activated Torso receptor is transmitted through a classical MAP-Kinase pathway, leading to phosphorylation and degradation of Capicua and allowing expression of zygotic genes, *tailless* and *huckebein* in restricted domains (adapted from Duncan et al., 2014).

1.17 The dorso-ventral system

In contrast to the specification of the antero-posterior axis specification that relies on three systems, a single system specifies the embryonic identity along the dorso-ventral axis (DV).

The cells derived from the most dorsal region of the embryo produce cuticle bearing dorsal hairs. Dorso-laterally derived cells secrete naked cuticle lacking specific structures except at the posterior end of the larvae where they form the tracheal spiracles and the Filzkörpers. Cells originating in ventrolateral regions generate the conspicuous band of ventral denticles that aid the larvae in moving through food (Fig.16A). In embryogenesis, loss of proper DV patterning causes gastrulation movements to occur all around the circumference of the embryo rather than on the dorsal side as in *wild type* embryos (Stein and Stevens, 2014).

Genetic screens for maternal effect mutations that affect embryonic patterning led to the identification of an entire class of genes named as the "dorsal" group (*dorsal, easter, gastrulation defective, nudel, pelle, pipe, snake, spätzle, Toll, tube, windbeutel*) as their loss of function mutation caused the lack of ventral denticles and the embryos display dorsal hairs all around its circumference (Fig.16B) (hence defined as "dorsalised") (Nusslein-Volhard and Wieschaus, 1980)). On the other side, it was found that loss of function mutation in the *cactus* gene led to an expansion of ventral denticle bands all around the embryos circumference (hence defined as "ventralised") (Roth et al., 1991).

Genetic anlaysis of *Toll* allowed the identification, in addition to loss-of-function mutations that give rise to dorsalised embryos, of dominant gain-of-function mutations that resulted in ventralised embryos (Anderson et al., 1985a). These findings put *Toll* as the central element of the dorso-ventral system (Anderson et al., 1985a). The use of gain of function *Toll* alleles allowed for epistatic analysis to identify genes that were acting upstream and downstream of *Toll* (Anderson et al., 1985b; Schneider et al., 1991).

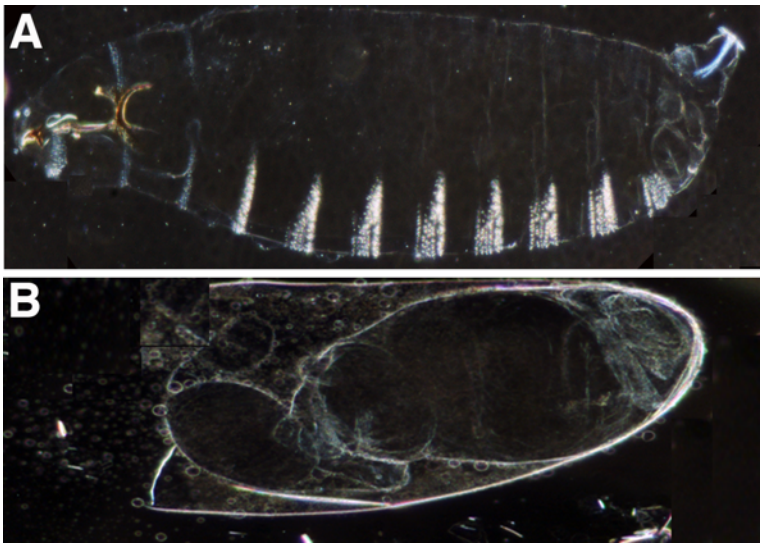

Figure.16 Cuticular structures specified by the dorso-ventral system A) Cuticle of a *wild type* embryo. **B)** Cuticle of an embryo displaying a dorsalised phenotype. Note the lack of ventral denticles, mandibular structures and dorso-lateral structures as the spiracles and filzkörpers.

Indeed, it was found that *nudel, pipe, windbeutel, gastrulation defective, snake, easter* and *spätzle* were involved in the activation of Toll, while, *pelle, tube, cactus and dorsal* were involved in the signal transduction cascade downstream of Toll (Anderson et al., 1985b). Among the dorsal group genes, three of them are expressed during oogenesis by follicle cells (*nudel, pipe, windbeutel*) while the others are expressed in the germline (Nusslein-Volhard and Wieschaus, 1980).

1.18 Upstream of Toll

The cloning of *Toll* revealed that it encoded for a transmembrane receptor uniformly distributed all along the plasma membrane of the syncytial blastoderm stage embryo (Hashimoto et al., 1991; Hashimoto et al., 1988). These data suggested that Toll bound a ligand that is restricted to the ventral region of the perivitelline space, the space between the vitelline membrane and the embryo plasma membrane, thus restricting Toll activation exclusively on the ventral side of the embryo (Morisato and Anderson, 1994; Schneider et al., 1994). Molecular characterization of additional members of the dorsal group provided evidence for a protease cascade acting upstream of Toll. Indeed, it was found that *gastrulation defective, snake* and *easter* encode

trypsin-like serine proteases expressed in the germline as inactive zymogens (DeLotto and Spierer, 1986; Chasan and Anderson, 1989)

Later studies showed that the *spätzle* gene encoded for the Toll ligand (Morisato and Anderson, 1994; Schneider et al., 1994).. Indeed, injection of a truncated form of *spätzle* mRNA containing the carboxy-terminal 106 amino acids is able to activate Toll in embryos laid by females carrying mutation in any of the dorsal group genes, while embryos from Toll mutant mothers remained dorsalised (Schneider et al., 1994). These data indicated that a cleaved form of the Spätzle protein constituted the active Toll ligand (Schneider et al., 1994). Moreover epistatic analysis supported a protease cascade model in which Gastrulation Defective acts on Snake, Snake on Easter, and Easter cleaves Spätzle (Dissing et al., 2001; LeMosy et al., 2001). However, *gastrulation defective, snake* and *easter* are expressed in the germ line while genetic data suggested that Toll was activated ventrally, therefore, a mechanism must exist to restrict the Spätzle cleavage exclusively on the ventral side of the embryo. The characterisation of the *pipe* locus demonstrated that it represents the critical link between follicular epithelium and restricted Toll activation (Sen et al., 1998; Stein and Stevens, 2014). As mentioned above, *pipe* is transcribed in a spatially restricted domain of ventral follicle cells due to the activation of Torpedo (Andreu et al., 2012; Atkey et al., 2006). The *pipe* gene encodes a Drosophila orthologue of two vertebrate enzymes, heparan sulfate 2-O-sulfotransferase (HSST) and dermatan/chondroitin sulfate 2-O-sulfotransferase (D/CSST) (Sen et al., 1998; Sergeev et al., 2001). Both enzymes, as well as Pipe, reside in the Golgi apparatus. Golgi localization of Pipe requires the chaperone activity of the chaperone protein Windbeutel (Sen et al., 2000).

During oogenesis, Pipe sulfates many vitelline membrane proteins and, in particular, a vitelline membrane protein named Vitelline Membran-Like (VML) (Zhang et al., 2009a). Thus, VML and other vitelline membrane components sulfated by Pipe are incorporated into the forming vitelline membrane where they would act as a ventrally localized cue that give rise to the protease cascade required to generate the active form of the Toll ligand (Zhang et al., 2009a). Indeed, in embryogenesis, Gastrulation Defective interacts with Pipe-sulfated proteins that are included in the ventral vitelline membrane (Cho et al., 2012).

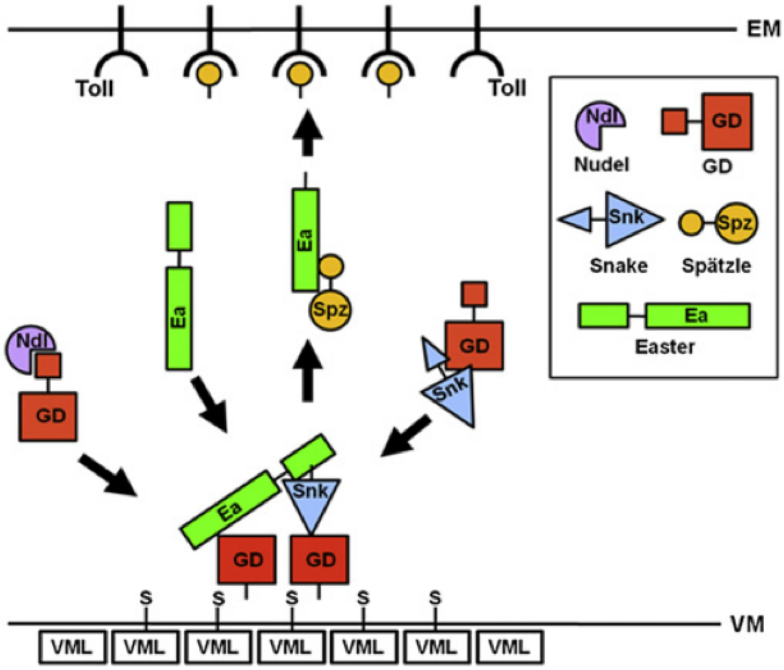

Figure.17 Model for restricted activation of Spätzle ventrally. Gastrulation defective (GD in red) is probably processed by Nudel (Ndl in violet). GD cleaves Snake (Snk in blue). Processed GD binds to Pipe-sulfated (S) targets (as Vitelline Membrane-Like VML) in the ventral part of the vitelline membrane and brings together Snk and Eastern zymogen (Ea in green), facilitating Ea cleavage. Processed Ea cleaves Spätzle (Spz in yellow) that binds and activates Toll in the ventral embryonic membrane (EM) (adapted from Cho et al., 2012).

This interaction enhances the ability of Gastrulation Defective to facilitate the cleavage of Easter by activated Snake, resulting in ventrally restricted processing and activation of the Easter protein (Cho et al., 2012). In this way, active Eastern is generated ventrally guaranteeing restricted Spätzle cleavage and localised Toll activation (Fig17) (Stein and Stevens, 2014).

However, in the protease cascade model described above, the role of *nudel* remains poorly understood and will be discussed in the next paragraph.

1.19 Nudel

The *nudel* (*ndl*) gene encodes for a very large secreted protein of 2616 amino acids with a central domain that exhibits homology to trypsin-type serine proteases (Hong and Hashimoto, 1995). *ndl* mutant alleles fall into two phenotypic classes. Eggs laid by

females carrying Class I alleles (associated with a reduction or complete absence of the Ndl protein, truncation, or deletion of portions of the protein, or altered processing, secretion or stability of the full-length protein (LeMosy et al., 2000) collapse or fail to sustain embryonic development. Embryos from females carrying Class II alleles (consisting in missense mutations in the serine-protease domain (Hong and Hashimoto, 1996) are able to complete embryogenesis but are dorsalised. Moreover, in these embryos the vitelline membrane is soft and unusually permeable to lipophilic dyes such as neutral red due to a failure of the non-disulphide cross-linking (LeMosy and Hashimoto, 2000). Lots of heteroallelic combination between Class I and Class II alleles give rise to embryos that are dorsalised and display defects in the vitelline membrane cross-linking in a temperature sensitive manner, as their penetrance increases with temperature (LeMosy and Hashimoto, 2000). However, some specific combinations of Class I with Class II alleles are capable of complementing one another, leading to the production of *wild type* larvae and adults (LeMosy et al., 2000). In mid-oogenesis, Ndl is expressed and secreted by all follicle cells and accumulates at the oocyte plasma membrane (fig.18B) although it has been also identified as a component of the eggshell (Fakhouri et al., 2006; LeMosy et al., 1998). In embryogenesis, Ndl localises at the embryo plasma membrane (fig.18C) (LeMosy et al., 1998).

Ndl undergoes a series of complex proteolytic processings. During oogenesis, Ndl is secreted as a 350 kD full-length protein that is processed in late oogenesis to form a 210 kD amino-terminal fragment that is, then, further processed generating a 170kDa polypeptide) (LeMosy et al., 1998). On the orther side, Ndl processing gives rise to a 250 kD carboxy terminal fragment, which contains the protease domain (LeMosy et al., 1998). At the moment of egg activation, the 250 kDa carboxy terminal fragment is processed further, first generating a 38 kDa fragment containing the protease domain and 110–130 kDa carboxy terminal (LeMosy et al., 1998; LeMosy et al., 2000). The Ndl 38 kD fragment undergoes an autoproteolytic cleavage generating the Ndl mature 33 kDa protease fragment and, at the same time, it cleaves the 110–130 kDa generating a 50–60 kD polypeptide (Fig.18A) (LeMosy et al., 1998).

Missense mutations in the protease domain (Class II alleles) cause the lack of Ndl autoproteolytic cleavage suggesting that Nudel provides a proteolytic function involved

in embryonic dorsoventral axis specification and vitelline membrane crosslinking. It has been suggested that a likely role for the Nudel protease in vitelline membrane cross linking might be the proteolytic activation of a cross-linking enzyme (peroxidase) or that Nudel protease could be involved in another aspect of the cross linking reaction, such as the release of H_2O_2 (LeMosy and Hashimoto, 2000). However, since females lacking of Ndl proteolitical activity lay eggs with defects in the vitelline membrane cross linking but that do not collapse, Ndl might have an additional structural function of Ndl in vitelline membrane integrity independent of its protease activity (LeMosy and Hashimoto, 2000; Stein and Stevens, 2014). One possibility might be that Nudel is required only for vitelline membrane cross-linking, with the Class I phenotype representing a complete loss of cross linking while the Class II would represent only a partial loss (LeMosy and Hashimoto, 2000). Alternatively, there might be a structural requirement for a non-protease region of Nudel during oogenesis and, accordingly, Class I *ndl* mutant egg chambers display modest structural abnormalities (unpublished observation in (LeMosy and Hashimoto, 2000).

Ndl is extensively glycosylated and its glycosylation is essential for its function (Turcotte and Hashimoto, 2002). This finding led to the hypothesis that glycosaminoglycans bound to Nudel might undergo Pipe-mediated sulfation (Stein et al., 2008). Thus, although uniformly localised at the oocyte membrane, Ndl would be active ventrally upon the activity of Pipe. However, clones homozygous for *ndl* mutations (using either Class I or Class II alleles) in the ventral follicle cells do not lead to local disruptions in embryonic patterning or vitelline membrane integrity showing that Ndl is not specifically required ventrally (Nilson and Schupbach, 1998; Stein et al., 2008).

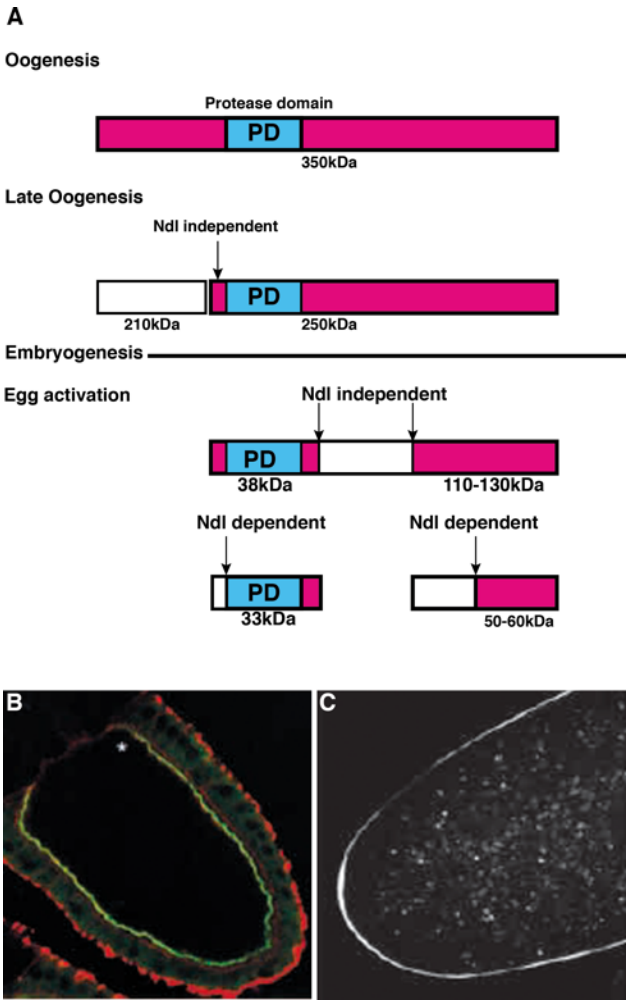

Figure.18 Nudel processing and localisation A) Schematic diagram of Nudel processing defined by Western blots. During oogenesis, Nudel is secreted as a 350 kDa full-length protein that is cleaved into a a 210 kDa N-terminal fragment and a 250 kDa C-terminal fragment containing the protease domain in late oogenesis. In embryogenesis, Nudel-independent processing occurrig probably at egg activation separates the protease domain (38kDa) from the C-terminal polypeptide and yields intermediate forms of the C-terminal polypeptide (110-130kDa). Ndl protease dependent processing gives rise to a putative active Nudel protease (33 kDa) and further processing of the remainder of Nudel C-terminal polypeptide (50-60 kDa). **B)** Stage 10A egg chamber stained with C-terminal Nudel antibody (green) and rhodamine-phalloidin (red) showing Ndl accumulation at the oocyte surface. Asterisk indicates the dorsoanterior position of the oocyte nucleus **C)** Early embryo stained with Ndl C-terminal antibody showing that Ndl is uniformly distributed on the embryo plasma membrane (all images were adapted from LeMosy et al., 1998).

Embryos laid by Class II *ndl* mutant flies fail to process Gastrulation Defective (GD) suggesting that it might be a substrate of Ndl protease activity (Cho et al., 2010;

LeMosy et al., 2001). However, Snake also fails to be cleaved in a *ndl* mutant background and this is not due to the lack of processing of GD as its protease activity does not require its processing (Cho et al., 2012; Cho et al., 2010; Steen et al., 2010). These data led to two alternative models for Ndl protease function. In the first one, called "two pathways model", the Nudel protease would act in two distinct pathways, one for the cross-linking of the vitelline memabrane and the other for the specification of the embryonic dorso-ventral axis. According to this model Nudel protease would act on two substrates, one involved in the cross-linking of the vitelline membrane (as a peroxidase) and the other in the dorso-ventral protease cascade (LeMosy and Hashimoto, 2000). In this scenario, the involvement of the Nudel protease in eggshell biogenesis is independent of its role in dorsoventral patterning.

The second model, called "one pathway model", envisage a scenario where correct dorso-ventral axis specification would be dependent upon the activity of Ndl in the cross linking of the vitelline membrane (LeMosy and Hashimoto, 2000). Ndl would be required for the formation of a correct vitelline membrane and, thus, the correct localisation of Pipe-mediated sulfated vitelline membrane components that activate GD (Cho et al., 2012; LeMosy and Hashimoto, 2000). Therefore, according to this hypothesis, Ndl would not be directly involved in the processing of a protease zymogen but would have an indirect influence on the proteolytic cascade in the perivitelline space (LeMosy and Hashimoto, 2000; Stein and Stevens, 2014).

1.20 Downstream of Toll

Epistasis analysis of the dorsal group genes revealed that three of its members, *dorsal*, *pelle, tube* and *cactus* act downstream of *Tl* (Anderson et al., 1985b).

Molecular charachterisation of *dorsal* revealed that it encoded a transcription factor displaying a high similarity to the mammalian transcription factor NFκB involved in the immune response and many other developmental processes (Ghosh and Baltimore, 1990). Toll signaling regulates Dorsal at the level of nuclear localization (Steward et al., 1988). Translocation of Dorsal to the nucleus is prevented by Cactus, a Drosophila homologue of the vertebrate IκB proteins that regulate NFκB activity (Geisler et al., 1992; Roth et al., 1991). Later studies reveled that genes belonging to the "dorsal

group" such as *tube*, *pelle* and many others are required to phosphorylate Cactus and sending it to degradation (Daigneault et al., 2013; Galindo et al., 1995; Towb et al., 1998). In this way ventrally restricted Toll activation is transduced into a gradient of nuclear localization of Dorsal in the embryo (Fig.19) (Roth et al., 1989). At the syncytial blastoderm stage, highest levels of nuclear Dorsal protein are present at the ventral side with successively lower levels in ventrolateral and dorsolateral nuclei and no Dorsal protein detectable in nuclei on the dorsal side (Roth et al., 1989). Nuclear concentration of Dorsal along the embryonic dorso-ventral axis defines the transcriptional state of specific zygotic genes (Roth et al., 1989). Indeed, the arrangement and number of Dorsal binding sites influence the affinity of Dorsal binding.

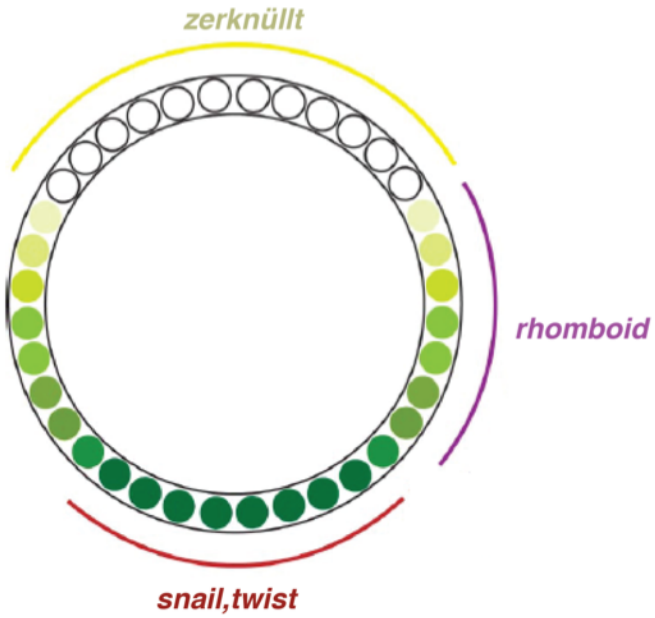

Figure.19 The Dorsal gradient. The Dorsal nuclear gradient regulates gene expression in a concentration-dependent manner across the DV axis of the early embryo. The expression domains of four of these genes are depicted on a diagram representing a cross-section through an early embryo. Filled green circles represent high levels of nuclear Dorsal protein, and shaded green and yellow circles represent intermediate and low levels, respectively. High levels of Dorsal activate genes such as *snail* and *twist* in the ventral-most embryonic region. Other genes such as *rhomboid* are activated by intermediate levels of Dorsal in ventro-lateral regions. In the most dorsal region, very low levels of nuclear Dorsal are not sufficient to repress genes as *zerknüllt* which is repressed in ventral and lateral regions (adapted from Stathopoulos and Levine, 2004).

Therefore, zygotic genes as *snail* and *twist* are activated only ventrally as their promoters bear low affinity binding sites for Dorsal and are activated only in cells containing high levels of Dorsal. Other genes such as *rhomboid*, are expressed in the ventral domain of the neurogenic ectoderm, where nuclear Dorsal is present at intermediate levels. Finally, in the most dorsal region of the embryo, very low levels of nuclear Dorsal are not sufficient to repress genes as *zerknüllt* (*zen*) which is repressed in ventral and lateral regions (Fig.19) (Stathopoulos and Levine, 2002a; Stathopoulos and Levine, 2002b). In this way different genes are expressed along the dorso-ventral axis specifying specific structures together with the genes expressed along the antero-posterior axis.

1.21 Torso signaling beyond the terminal system

As mentioned before, holometabolous insects develop through a series of larval stages called instars, in which the old cuticle is shed and replaced by a new larger one. At the end of the third instar (L3), when the larva has accumulated enough mass and stored sufficient nutrients, the larva pupate and metamorphosis takes place transforming the juvenile larva into a sexually mature adult by the destruction of larval tissues and the replacement by adult ones (Mirth and Riddiford, 2007). The transition through the first two larval stages is induced by a single pulse of ecdysone while the onset of pupariation (onset of metamorphosis) is triggered by three low level pulses followed by a high-level pulse at the end of L3 (Yamanaka et al., 2013a). Ecdysone is produced and released from the prothoracic gland (PG), the major endocrine tissues of insects, in response to a brain derived neuropeptide called prothoracicotropic hormone (PTTH) (McBrayer et al., 2007).

In the PG, it has been found that PTTH binds to the Torso receptor that, in turn, triggers the mitogen-activated protein kinase (MAPK) pathway to stimulate the production and release of ecdysone (Fig21A-B) (Rewitz et al., 2009). Indeed, ablation of PTTH expressing neurons or downregulation of *tor*, *ras* or *raf* in the PG causes a delay in reaching the onset of pupariation and give rise to large pupae and adults. Conversely, gain of function mutation in *ras*, (namely ras^{V12}) that constitutively activate the pathway in a ligand independent way, slightly anticipate the onset of

pupariation and give rise to small pupae and adults (Rewitz et al., 2009). Therefore, the Tor receptor is used beyond the embryonic terminal system binding to a different ligand, PTTH. Alignment of the protein sequences of Trk and PTTH reveals that they are paralogues as they share some conserved structures in the C-terminal region that compose the mature peptide including six cysteines (cysteine knot) that are important for intramolecular disulfide bonds (Fig.20) (Ishibashi et al., 1994; Rewitz et al., 2009).

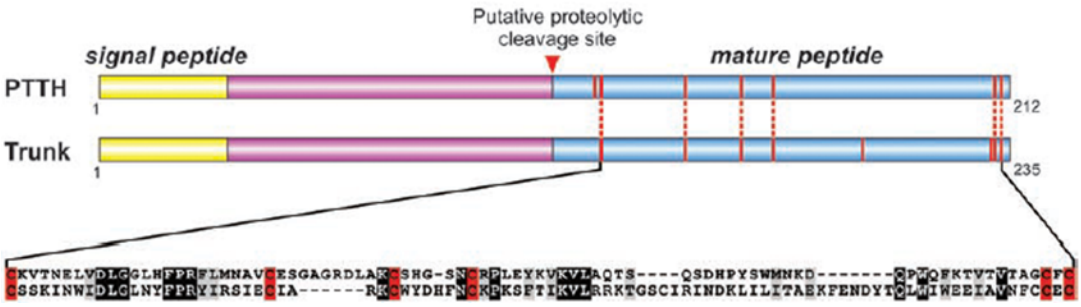

Figure.20 Sequence similarity between PTTH and Trk. Amino-terminal signal peptide in yellow, putative proteolytic cleavage site for both ligands is indicated by an arrow, red vertical lines indicate cysteines and numbers refer to amino acid residue (adapted from Rewitz et al., 2009).

Moreover, ectopic expression of PTTH in the embryo is able to rescue the lack of Trk reinforcing the notion that PTTH is a Tor ligand (Rewitz et al., 2009).

In the Lepidopteran *Bombyx mori*, it has been found a cleaved form of PTTH containing 109 carboxy-terminal residues that, probably, constitutes the PTTH mature form (Kawakami et al., 1990). Indeed, between the signal peptide and the mature form, PTTH contains in its sequence a variety of dibasic and tribasic amino acid patterns that are recongnised by pro-protein convertases as Furins (Kawakami et al., 1990; Smith and Rybczynski, 2011). Therefore, some insects apparently co-opted the same signaling pathway with two distinct ligands for developmentally distinct processes: the terminal system in the embryo and the regulation of developmental timing.

In later studies, it was found that *tsl* was also involved in regulating developmental timing as *tsl* mutant larvae had a delay in the onset of pupariation (Grillo et al., 2012). Moreover, specific downregulation of *tsl* in the PG lead to a delay in the onset of pupariation suggesting that Tsl was likely to play a specific role in the PG (Fig.21B).

In the PG of *wild type* larvae Tor activation triggers the di-phosphorylation of MAPK/ERK (dpERK), while, in contrast, no dpERK signal could be detected in the PG of *tsl* mutant larvae (Grillo et al., 2012). Conversely, *fs(1)N, fs(1)ph* or *clos* are not expressed in the PG and do not affect developmental timing suggesting that not all the components of the terminal system have a conserved role in the PG (Grillo et al., 2012). From these data, it was hypothesised that Tsl in the PG had a role in activating PTTH similar to its role in the embryo. Accordingly, ectopic overexpression in the PG of $Trk^{C\text{-}108}$, a truncated version of the protein that acts as an active form of Trk in embryonic patterning (see above) (Casali and Casanova, 2001), has a mild but significant effect in advancing the time of pupariation (Grillo et al., 2012).

However, further studies suggested that Tsl is not likely to play any role in the PG. On the contrary, it was proposed that Tsl acted independently of Tor in regulating developmental timing and body size (Johnson et al., 2013). Accordingly, *tor;tsl* double mutants had a dramatic delay in reaching pupariation showing that *tsl* had an additive, rather than epistatic, effect on *tor* (Johnson et al., 2013). Although Tor and Tsl are both involved in regulating developmental timing, they have opposite effect on body size regulation. While downregulation of *tor* in the PG or ablation of PTTH-producing neurons results in larger adults due to prolonged duration of feeding as larvae (McBrayer et al., 2007; Rewitz et al., 2009), *tsl* mutants or *tor;tsl* double mutants are smaller than *wild type* larvae (Johnson et al., 2013). Moreover, it was shown that Tsl does not play a role in the PG as *tsl* overexpression in the PG does not rescue either the developmental delay or the body size observed in *tsl* mutant larvae (Johnson et al., 2013).

Further evidences suggested that PTTH does not require Tsl either in the PG or the embryo. Indeed, ectopic PTTH expression in the germ line is able to ectopically induce Tor activation even in the absence of *tsl*. This result suggest that, in the embryonic terminal system, while Tsl acts on Trk leading to the restricted Tor activation at the poles, PTTH is already secreted as an active ligand that does not require Tsl to activate Tor (Johnson et al., 2013).

Finally, it was found that a HA tagged form of Tsl was able to rescue the reduction in body size and the delay in reaching pupariation of *tsl* mutant larvae although it was not able to be functional in the context of embryonic terminal pathway corroborating the

hypothesis that Tsl might have different modes of action; one in the embryo terminal system that depends on Tor and the other in developmental timing and body size that is Tor independent (Johnson et al., 2013).

Torso and PTTH have been shown to have further roles besides the induction of ecdysone in the PG. It has been shown that PTTH is secreted into the haemolymph and has an endocrine function in regulating light avoidance behaviour (Yamanaka et al., 2013b). Indeed, PTTH activates Tor signalling in the Bolwig'sorgan (BO) and in the class IV dendritic arborisation (da) neurons tiling the larval body wall (Yamanaka et al., 2013b), neuronal populations previously identified as light sensors in Drosophila (Fig21A) (Keene and Sprecher, 2012). Specific loss of PTTH/Tor signalling in these neuronal populations abolishes light avoidance. Importantly, the light avoidance behaviour mediated by PTTH/Tor signalling ensures that wandering larvae have a dark preference for their pupariation site, probably providing protection from predators and dehydration during the immobile pupal stage (Yamanaka et al., 2013b). Therefore, the PTTH/Tor signalling has a dual role in the larval developmental promoting the induction of pupariation and triggering innate behaviour that might increase the chances to survive to metamorphosis (Yamanaka et al., 2013b).

Recently, Tor has also been identified in the larval fat body, an organ that has endocrine and storage functions and is the functional equivalent of the mammalian adipose tissue and liver (Fig21A) (Danielsen et al., 2013; Jun et al., 2016). Specific down regulation of Torso in the fat body cause a reduction of the pupal size and suppresses Insulin/TOR signalling (Jun et al., 2016). Thus, Tor activity leads to an increase or decrease in final pupal size according to the tissues where is activated.

In summary, the Tor receptor work as crucial element in the larvae to coordinate larval growth, timing and behaviour of pupariation.

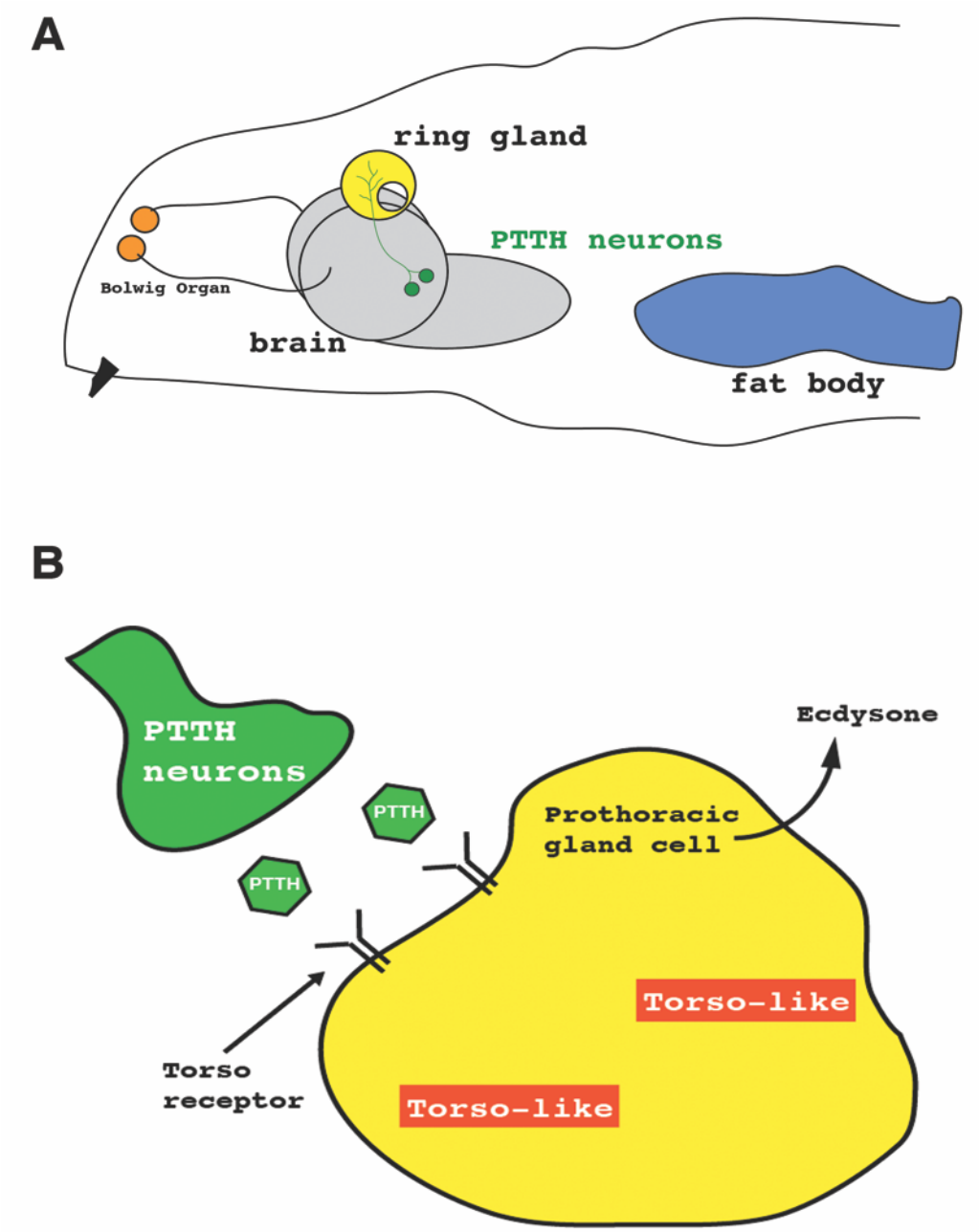

Figure.21 Torso receptor expression beyond the terminal system A) In the third instar larvae, Tor is expressed in the ring gland (in yellow) innervated by PTTH producing neurons (in green) from the brain (in grey). Tor is also expressed in the Bolwig Organ (in orange), in class IV dendritic arborisation neurons (not shown) and in the fat body (in blue). **B)** PTTH is secreted by PTTH neurons and interacts with the Tor receptor at the membrane of the cells of the Prothoracic gland. Interaction between PTTH and Torso activates the MAP-Kinase pathway leading to ecdysone synthesis and release. Tsl is also expressed by prothoracic gland cells and it is probably required for ecdysone release.

Objectives

2. Objectives

The mechanisms that lead to the restricted activation of Tor at the embryo poles receptor are not fully characterised. In particular, although the Tsl, Nasrat, Polehole and Closca proteins are known to be required for Tor activation, their role in the terminal system remains elusive. The main objective of this work was to shed light on the role of these proteins in ensuring restricted of Tor activation at the embryonic poles.

1. Among the genes identified in Tor signalling in the embryo, *tsl* is the only one expressed at the poles of the egg chamber. In embryogenesis, Tsl is anchored at the poles in the inner side of the vitelline membrane. However, it remains an open question how Tsl at the vitelline membrane influences Tor activation. Moreover, Tsl bears a membrane-attack complex/perforin domain (MACPF), a domain present in proteins associated with the plasma membrane. Since this observation was difficult to reconcile with Tsl function at the vitelline membrane, we analised Tsl localisation in oogenesis and embryogenesis to elucidate its mechanism of action in the embryonic terminal signalling.

2. A proper vitelline membrane crosslinking plays a crucial role for embryonic axis specification. Although the mechanism that trigger the cross linking are not known, the Nasrat, Polehole and Closca on the one side and the Ndl protease on the other are known to be required for this process. We investigated the functional relationship among them to elucidate their role in embryonic patterning and the transfer of positional information from the ovary to the embryo.

Materials and Methods

3.1 Fly stocks

Mutant Alleles	Class	Description	Reference
tor^{XR1}	amorph	9.5kb deletion within the *tor* locus.	(Sprenger et al., 1989)
tsl^{604}	amorph	P-element- insertion in the *tsl* promoter	(Martin et al., 1994)
tsl^{Δ}	amorph	deletion of *tsl* coding region	(Johnson et al., 2013)
$Df(3R)caki^{X-313}$	amorph	chromosomal deletion covering the *tsl* locus	(Martin et al., 1994)
tsl^{1}		Amino acid replacement: V215M.	(Savant-Bhonsale and Montell, 1993)
tsl^{3}		Amino acid replacement:Y279N	(Savant-Bhonsale and Montell, 1993)
tsl^{2}		Amino acid replacement:D109V	(Savant-Bhonsale and Montell, 1993)
tsl^{5}		Amino acid replacement:Y148N	(Savant-Bhonsale and Montell, 1993)
sra^{KO}	amorph	Part of the *sra* gene has been deleted	(Takeo et al., 2006)
sra^{A426}	strong hypomorph	P-element insertion in intron of the *sra* gene	(Horner et al., 2006)
trk^{3}	amorph	Point mutation introducing a stop codon within the coding sequence, resulting in a truncated protein	(Casanova et al., 1995)
$fs(1)N^{14}$	amorph	Small deletion of putative promoter and first exon sequences of the *fs(1)N* locus.	(Jimenez et al., 2002)
$fs(1)N^{12}$		Amino acid replacement: P830L. Terminal allele	(Jimenez et al., 2002)
$fs(1)N^{4}$		Behaves as null but is fully complemented by $fs(1)N^{211}$	(Degelmann et al., 1990)
$fs(1)ph^{1901}$		Amino acid replacement: Y742N Terminal allele	(Jimenez et al., 2002)
$fs(1)ph^{K646}$	amorph	Considered as amorph by genetic analysis	(Jimenez et al., 2002)
$clos^{1}$		Amino acid replacement: V509D. Terminal allele	(Ventura et al., 2010)
$clos^{4152}$		P-element insertion in the *clos* coding sequence	(Ventura et al., 2010)
ndl^{111}		Nucleotide substitution: G4268A within the catalytic domain. *ndl* Class II allele	(LeMosy and Hashimoto, 2000)
ndl^{14}	amorph	Nucleotide substitution: W1044 is changed into a stop codon within the coding sequence prior to the protease domain. *ndl* Class I allele	(LeMosy et al., 2000)
Df(3L)CH12	amorph	Chromosomal deletion covering the *ndl* locus	(Hong and Hashimoto, 1995)
$Vm26Ab^{QJ42}$		mRNA levels of *sV23* are strongly reduced and the protein is not detectable	(Savant and Waring, 1989)
Df(2L)BSC183	amorph	Chromosomal deletion covering the *Vm26Ab* locus	(Savant and Waring, 1989)

We used the following *Drosophila* stocks described in Flybase: *yw* flies as *wild type.* In all the experiments we employed the following transheterozygous combinations:

sra^{A426}/sra^{KO}, $clos^{1}/clos^{4152}$, ndl^{111}/Def(3L)CH12, ndl^{14}/Def(3L)CH12, $Vm26Ab^{QJ42}$/Def BSC183.

Gal4 drivers	Insertion	Description	Ref
Pg45	GAL4-VP16 fusion protein insertion in the *myc* promoter	Gal4 expression ubiquitously	(Bourbon et al., 2002)
mat-tub	GAL4-VP16 fusion protein expressed under the control of the *alphaTub67C* promoter	Gal4 expression through oogenesis and loaded into eggs	(Staller et al., 2013)
tub-P	GAL4-VP16 fusion protein expressed under the control of the *alphaTub84B* promoter	Gal4 expression ubiquitously	(Lee et al., 1999)
Cy2	Not known	Gal4 expressed in all follicle cells starting from stage 8 onward	(Queenan et al., 1997)

Transgenic lines	Expression domain	Descripion	Ref
UAS-t-Torso-like	UAS	*tsl* full cDNA sequence downstream of UAS promoter	(Stevens et al., 2003)
UAS-p-PTTH-HA	UAS	overexpression of *ptth* full cDNA sequence fused with a HA tag at C-terminus	(Johnson et al., 2013)
UAS-VML-RFP	UAS	Vitelline-membrane like protein fused with mRFP at the C terminus	(Jimenez et al., 2002)
Polehole-HA	endogenous promoter	*fs(1)ph* full cDNA sequence fused with a HA tag at C-terminus	(Jimenez et al., 2002)
Tsl-HA	endogenous promoter	*tsl* full cDNA sequence fused with a HA tag at C-terminus	(Jimenez et al., 2002)
Nasrat-HA	endogenous promoter	*fs(1)N* full cDNA sequence fused with a HA tag at C-terminus	(Jimenez et al., 2002)

3.2 Preparation of embryonic cuticles

In order to detect embryonic phenotypes, we analysed the embryonic cuticular structures as described by (Wieschaus and Nusslein-Volhard, 1986). In every cxperiment, at least 120 embryos were scored.

Twenty four to 48h old embryos were collected with 0.1% Triton, dechorionated with bleach, washed with 0.1% Triton, mounted with Hoyer:lactic (1:1), and incubated at 50–60°C overnight. Dark field photographs were taken in a Zeiss (Oberkochen, Germany) Axioskop microsocope.

3.3 Neutral Red assay

Eggs in which the outer chorion layer has been experimentally removed are normally impermeable to small molecules such as Neutral Red, apparently due to the lipid wax layer covering the vitelline membrane (Margaritis 1980;Papassideri et al., 1993). Defects in the vitelline membrane cross linking disrupt this lipid wax layer leading indirectly to the permeability of the eggs to Neutral Red (Schlichting et al., 2006). Therefore we used this assay to detect defects in vitelline membrane cross linking. A minimum of 130 eggs were scored.

Flies were allowed to lay eggs for 0-12 hours in peach agar plates at different temperatures. Embryos were collected with Triton X-100 0,1%, dechorionated with bleach for 2 min, washed in water to remove bleach and transferred to an eppendorf containing Neutral Red 5mg/ml (Sigma) according to (LeMosy and Hashimoto, 2000). Embryos were incubated for 10 minutes with gentle rocking, washed 5 times with PBT (PBS-Tween 20 0,1%) and mounted in Fluoromount (Southern Biotech).

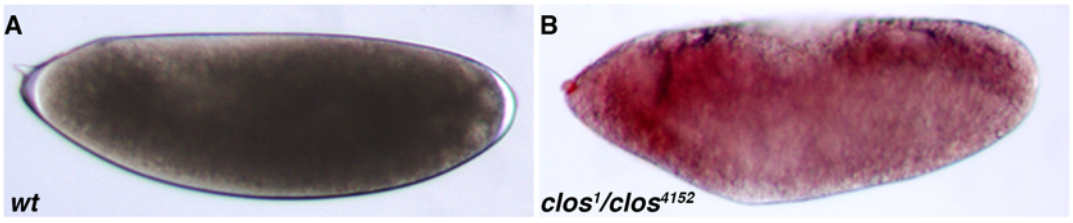

Figure.22 Neutral red assay. A) Dechorionated eggs from *wild type* and **B)** $clos^{1}/clos^{4152}$ females females incubated in a neutral red solution for 10 minutes. *Wild type* embryos are impermeable to the dye whereas most of the $clos^{1}/clos^{4152}$ eggs are stained with varying intensity.

3.4 Immunohystochemistry and Imaging analysis

All Tsl embryonic stainings were performed using heat-fixation as described previously (Tanentzapf et al., 2007; Tepass, 1996). Flies were allowed to lay eggs for 2 hours in peach agar plates at 25ºC or as otherwise stated. Embryos were collected with Triton X-100 0,1%, dechorionated with bleach for 2 min, washed in water to remove bleach, and then immersed in boiling E-wash buffer (100 mM NaCl, 0.3% Triton X-100) for a 8-12 seconds, then immediately cooled by adding 4 volumes of ice-cold E-wash and placed on ice for 1 minute. Afterwards, embryos were devitellinized in heptane-methanol 1:1 and collected in methanol.

Fixed embryos were extensively washed with PBT (PBS-Tween 20 0.1%) for 4-5 times and blocked with PBT-BSA (Bovine Serum Albumin) 0,5% for at least 20 minutes and incubated with primary antibodies in PBT-BSA 0,5% overnight at 4ºC. After removal of primary antibodies, embryos were washed with PBT for 4-5 times and blocked with PBT-BSA0,5% for 20 minutes, incubated with fluorescently conjugated secondary antibodies in PBT-BSA0,5% for 2 hours at room temperature and mounted in Fluoromount (Southern Biotech).

For ovary dissection, flies were placed in food vials supplemented with fresh yeast paste 1–2 d before dissection. For Tsl staining, ovaries were heat-fixed while in all other cases ovaries were fixed with 4% formaldehyde (see below).

Ovaries were collected in cold PBS, and then immersed in boiling E-wash buffer (100 mM NaCl, 0.3% Triton X-100) for a 8-12 seconds, then immediately cooled by adding 4 volumes of ice-cold E-wash and placed on ice for 1 minute. Afterwards, ovaries were immediately rehydrated with PBT (PBS-Tween 20 0.1%) for 4-5 times and blocked with PBT-BSA0,5% for at least 20 minutes and incubated with primary antibodies in PBT-BSA 0,5% overnight at 4ºC. After removal of primary antibodies, ovaries were washed with PBT for 4-5 times and blocked with PBT-BSA0,5% for 20 minutes, incubated with fluorescently conjugated secondary antibodies in PBT-BSA0,5% for 2 hours at room temperature and mounted in nPG antifade mounting media (glycerol 80% n-propyl-gallate 0.4%).

Alternatively, ovaries were dissected in cold PBS, fixed for 20 min in 4% formaldehyde with PBS 0.1% Triton X-100 (PBTr), rinsed 3–5 times with PBTr, blocked for 1 h with PBTr 10%BSA, and incubated overnight at 4ºC with the primary

antibodies in PBTr 1%BSA. Afterwards, ovaries were washed for 2 h with PBTr 1%BSA and incubated for 2h with fluorescently conjugated secondary antibodies in PBTr 0.1%BSA, washed with PBTr and mounted in nPG antifade mounting media (glycerol 80% n-propyl-gallate 0.4%).

The following primary antibodies were used: anti-Tsl 1:50 (Grillo et al., 2012), anti-Yl 1:1/500 (Schonbaum et al., 2000), anti-HA (12CA5 1/100 and 3F10 1/300, Roche), anti-Spectrin 1/5 (3A9, D.S.H.B.), anti-sV23 1/100 (Pascucci et al., 1996), anti C-Ndl 1:400 (LeMosy et al., 1998), anti-CVM32E 1/150 (Andrenacci et al., 2001), anti-RFP (ab62341,Abcam; 1/300) and secondary antibodies 1/300 (Jackson ImmunoResearch). Confocal images were obtained with a Leica SPE, analysed in Fiji and assembled with Adobe Photoshop. All images were oriented anterior to the left and dorsal up.

3.5 *In situ* Hybridization

Flies were allowed to lay eggs for 0-3 hours in peach agar plates at 29ºC. Embryos were collected and dechorionated as described above and incubated for 20 minutes in PBS:Heptane 1:1 containing 4% Formaldehyde. Afterwards, embryos were devitellinized in heptane-methanol 1:1 and collected in metanol.

In situ hybridization was performed according to (Tautz and Pfeifle, 1989).

After fixation with formaldehyde, embryos are first washed 5 times for 5 min each in PBT (PBS-Tween 20 0,1%). Embryos were then incubated in hybridization solution (HS) (50% formamide, 750 mM NaCl, 75 mM sodium-citrate, 50µg/ml heparin, 0.1% Tween 20 and 100 gg/ml sonicated and denatured salmon sperm DNA) for at least three hours at 56 ºC. Antisense RNA probes labeled with Digoxigenin conjugated Uracile were added at the concentration of 500ng/µl approximately. Before addition, the probe was boiled for five minutes and rapidly chilled on ice. Embryos were incubated with the probe overnight at 56ºC. After removal of the probe, embryos were washed 5 times at room temperature for 20 min at each step. being the first wash HS, the second HS-PBT 1:1 and the last 4 washes in PBT.

Embryos were then incubated with anti-Digoxigenin antibody conjugated with Alkaline Phosphatase (Roche) diluted 1:2000 in PBT at room temperature for two hours. After the antibody was removed, embryos were washed four times with PBT for 20 minutes at each step each. Embryos were then trasferred to a multiwell dish and

incubated in Alkaline Phosphatase buffer (100 mM NaC1,50 mM MgC12, 100 mM Tris pH 9.5, 1 mM,0.1%Tween 20) containing 4.5 mg/ml NBT BCIP until an intense purple color was developed. Phosphatase alkaline reaction was stopped by a series of 5 washing steps with PBT. Embryos were then dehydrated in an ethanol series and mounted in Permount.

3.6 *In vitro* egg activation

For *in vitro* egg activation, isolation, activation, and recovery buffers were prepared as described by (Endow and Komma, 1997; Page and Orr-Weaver, 1997). Whole ovaries from females aged 4–6 days on yeast paste were used to enrich for mature Stage 14 oocytes that can respond to egg activation. The ovaries were dissected in isolation buffer (55 m NaOAc, 40 mM KOAc, 110 m sucrose, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$ M , 100 mM Hepes brought to pH 7.4 with NaOH) at room temperature. To activate Stage 14 oocytes, isolation buffer was removed and substituted by fresh activation buffer (3.3mM NaH_2PO_4 , 16.6 mM KH_2PO_4 , 10mM NaCl, 50 mM KCl, 5% PEG 8000, 2 mM $CaCl_2$, brought to pH 6.4 with 1:5 NaOH:KOH) over 5 min and homogenised as described above and analysed by Western blot. For Tsl immunostainings, in vitro activated oocytes were immediately heat-fixed and stained. Alternatively, activation buffer was removed and oocytes were incubated for 60 minutes in recovery buffer (Zalokar's buffer) (9 m M $MgCl_2$, 10 mM $MgSO_4$, 2.9 mMNaH_2PO_4, 0.22 m NaOAc, 5mM glucose, 27mM glutamic acid, 33 mM glycine, 2 mM malic acid, 7 mM $CaCl_2$, brought to pH 6.8 with 1:1 NaOH:KOH) demonstrated to support the oocyte development (Limbourg and Zalokar, 1973).

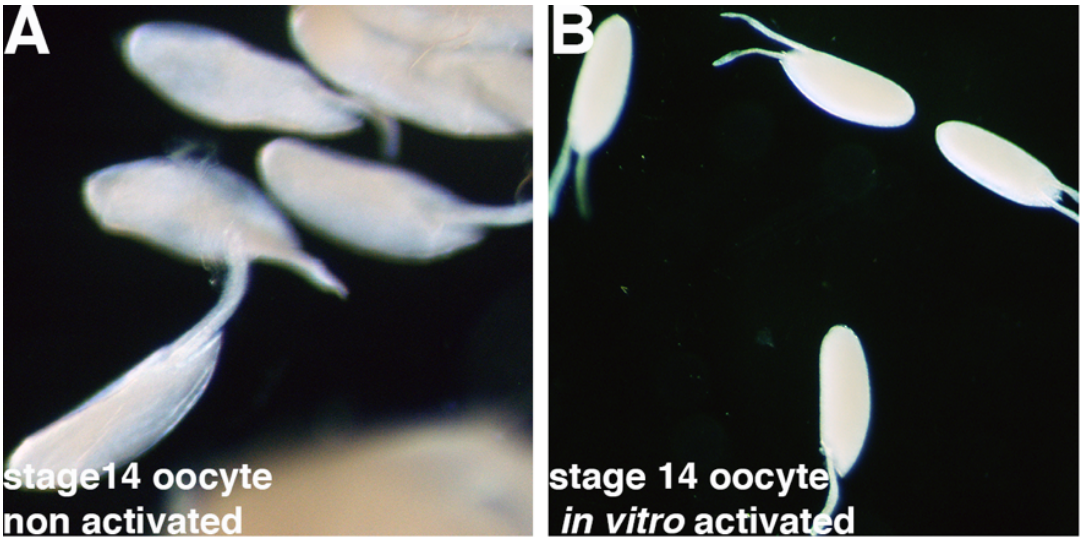

Figure.23 ***In vitro*** **egg activation** **A**) Stage 14 oocytes maintained in isolation buffer. **B**) stage 14 oocytes activated *in vitro*.

3.7 Western blot analysis

Ovaries were dissected in cold PBS solution and immediately homogenised. Embryos were collected on peach juice plates and washed extensively with water. Samples were homogenized on ice in 2XSDS sample buffer (Tris 1 M, pH 6.8, 50% glycerol and 10% SDS) containing 6 M urea and 100 mM DTT accordingly to (LeMosy et al., 1998) boiled for 5 minutes and soluble proteins loaded on 10% or 4-20% SDS-polyacrylamide gels (Amersham). Typically, 3 ovaries or 50-60 embryos from 0-1,5h old were loaded per lane. Proteins were transferred to PVDF membranes (Millipore), blocked for 1h with PBS: Odyssey blocking buffer (Licor) and incubated with primary antibodies overnight at 4ºC with PBS: Odyssey Blocking buffer 1:1. Membranes were washed with PBT (PBS-Tween 20 0,1%), incubated with seceondary antibodies in PBT for 1h at room temperature and analised using the Odyssey CLx imaging system (Licor). The following antibodies were used: anti C-Ndl 1:1000 (LeMosy et al., 1998), anti-Tubulin 1:5000 (Millipore) and secondary antibodies 1/10000 (IRDye 800 and 680 from Licor).

3.8 Synthesis of *zerknullt* (*zen*) RNA probe for *in situ* hybridization

A pGEM-T vector (Promega) containing a fragment of ~500 bp of the zerknullt cDNA

was kindly provided by Nùria Sampér and linearised using KpnI restriction enzyme and purified. Antisense RNA probe was obtained using T7 RNA polymerase (Roche), labelled with digoxigenin and purified with LiCl-ethanol mix. Probe concentration was measured using spectrophotometric analysis, run into an agarose gel and quantified to verify the efficiency of transcription.

3.9 Sequencing of the *fs(1)N*^{4} allele

Genomic DNA extraction

fs(1)N^4 mutant flies were homogenised in solution A (Tris-HCl 0,1M pH 9), EDTA 0,1M, SDS1%) incubated at 70ºC for 30 minutes. Afterwards, it was added 35 µl KAc, incubated on ice for 30 minutes, centrifugated at 15000g for 15 minutes. Supernatant was loaded into a new tube with one volume of Phenol-Chloroform, precipitated with isopropanol, washed with ethanol and resuspended in TE buffer.

PCR amplification and sequencing of the *fs(1)N*^{4} locus

Genomic DNA obtained from *fs(1)N*^{4} flies was used as template to amplify the coding sequence of the *fs(1)N* locus (~7kbp) using the following primers:

Fw primer: 5' AGTTTTTTCTTCGCATATCTGC 3'
Rv primer: 5' CTTTTTAAACATCACCAAACC 3'

PCR products were sequenced using internal primers, every ~600bp, within the *fs(1)N* coding sequence. Sequences were assembled using the EMBOSS software and analised with the MacVector Sequence Tools software. *fs(1)N*^{12} were sequenced as a control to identify polymorphisms in the *fs(1)N*^{4} strain.

Results

4.The Torso-like protein translocate from the vitelline membrane to the embryonic plasma membrane

4.1 Torso-like localisation at the embryonic plasma membrane

The Torso-like protein is the only MACPF domain containing protein in *Drosophila melanogaster* (Pointing 1999). As mentioned before the MACPF domain is a specific domain that has been found in proteins which are able to bind to the plasma membrane of cells which have been infected by a pathogen and trigger the innate immune response (Kondos et al., 2010).

In this scenario, Tsl localisation at the vitelline membrane did not fit with its protein features. Indeed, although only few of the MACP domain containing proteins are able to form pores, they have always been found associated to plasma membranes, whereas, on the other hand, Tsl has been reported to localise at the embryonic vitelline membrane (Stevens et al., 2003) which is constituted by different proteins tightly packed through covalent bonds (cross linking) and does not contain any phospholipid.

Therefore, we hypothesised that Tsl might be localised at the embryonic plasma membrane and, to confirm this hypothesis, we took advantage of an anti-Tsl antibody generated previously in the lab (Grillo et al., 2012). We confirmed that our antibody reproduced the *wild type* Tsl pattern in the egg chamber (Fig. 24A-A') and proved the specificity of the antibody by the absence of signal in the ovaries of females homozygous for tsl^{604} (Fig. 24B-B'), a mutation caused by the insertion of a P element in the promoter region of the gene that eliminates its function (Martin et al., 1994).

Nevertheless, we were unable to detect any specific signal when embryos were incubated with the anti-Tsl antibody upon formaldehyde or paraformaldehyde chemical fixation (data not shown). However, we could detect a clear Tsl accumulation at both embryonic poles when embryos were subjected to heat fixation (Fig.24 C-C'). Indeed, Tsl localised asymmetrically at the anterior pole and posterior poles of the syncytial and cellular blastoderm stage embryos.

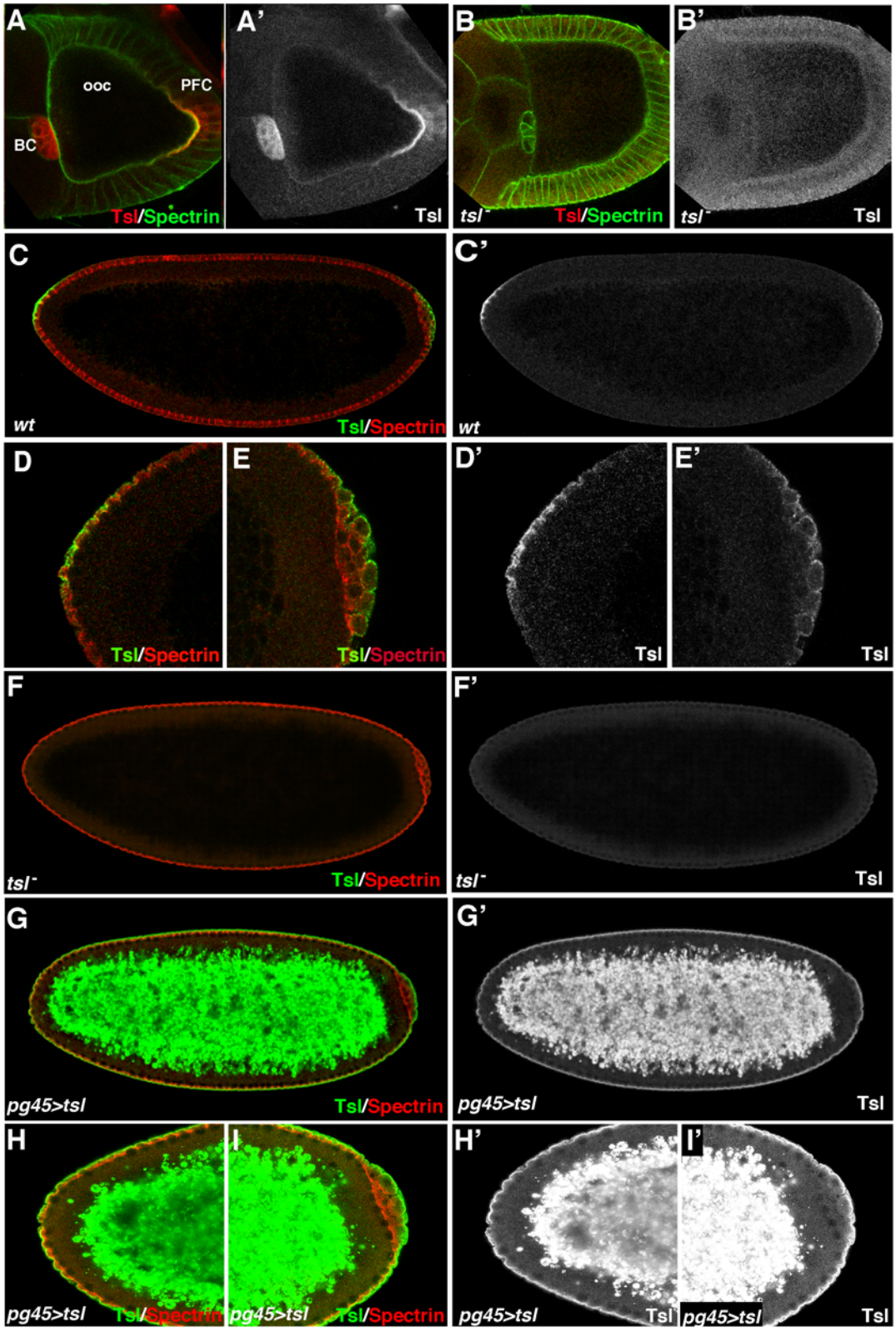

Figure.24 Tsl accumulates at the embryonic plasma membrane (**A**) Confocal section of a stage 10 egg chamber stained with anti-Tsl (red) and anti-Spectrin (green) antibodies. (**A'**) Same image in the red channel; antibody signal is detected in the cytoplasm of the border cells (BC) and the posterior follicle cells (PFC) and is also detected secreted extracellularly between the oocyte (Ooc) and the follicle cells.

(**B**) Confocal section of a stage 10 egg chamber from a *tsl* null mutant female (*tsl*^{604}) stained with anti-Tsl (red) and anti-Spectrin (green) antibodies. (**B'**) Same image in the red channel to show the specificity of the antibody staining. (**C**) Projection of confocal sections of a syncytial blastoderm embryo stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. (**D,E**) Magnifications of the same image including the anterior (D) and the posterior poles (E). (**C'-E'**) Same images in the green channel; antibody staining is detected at the poles of the embryonic plasma membrane. (**F**) Projection of confocal sections of a syncytial blastoderm embryo laid by a *tsl* null mutant female (*tsl*^{604}) stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. **F'**) Same image in the green channel to show the specificity of the antibody staining. **G**) Projection of medial confocal sections of a syncytial blastoderm embryo laid by a female overexpressing Tsl with the PG45-Gal4 driver in all follicle cells during oogenesis and stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. (**H,I**) Magnifications of the same image including the anterior (H) and the posterior poles (I). (**G'**) Same image as G in the green channel; Tsl is detected ectopically all around the embryonic plasma membrane. Note the high levels of Tsl inside the embryo (probably reflecting an uptake during oogenesis of the overexpressed Tsl together with yolk proteins). (**H',I'**) Same images in the green channel.

Therefore, we confirmed that the antibody signal at the embryonic plasma membrane was Tsl-specific as we could not detect any signal in embryos from *tsl*^{604} females (Fig. 24F-F') and, conversely, Tsl was found throughout the entire membrane upon its ectopic expression in all the follicle cells (Fig.24G-G'). Surprisingly we also found that upon *tsl* ectopic expression, high levels of Tsl accumulated within the embryos. This accumulation probably reflects an uptake during oogenesis of the overexpressed Tsl together with yolk proteins from the follicle cells to the oocyte. Alternatively, it might also be due to high turnover of the plasma membrane during early embryogenesis as it has been suggested in the case of Ndl (LeMosy et al., 1998).

To further confirm the specificity of Tsl accumulation, we quantified Tsl accumulation at the poles measuring the intensity of the signal detected at the poles compared to the one observed within the embryo (Fig.25A). We therefore obtained a curve that represents the intensity of the Tsl signal over the embryo length from anterior to posterior expressed in percentage from 0 to 100 (Fig.25B). With this analysis, we observed that although the intensity of the staining at the poles was variable between different embryos, it was consistent with a specific accumulation of Tsl at the poles. From this analysis it is also evident that the intensity of Tsl accumulation was higher at the posterior pole than in the anterior. The discrepancy between the two poles might be due to the difficulties in staining the anterior pole due to its sharp shape compared to the rounded shape of the posterior pole.

Nevertheless, we cannot rule out the possibility that lower levels of the Tsl protein are needed at the anterior pole to trigger proper Tor receptor activation. This might be explained as proper formation of anterior terminal structures depends on the Tor pathway but also on the anterior determinant *bicoid* and, then, lower Tsl levels are required to give rise to proper specification of anterior terminal strucutres.

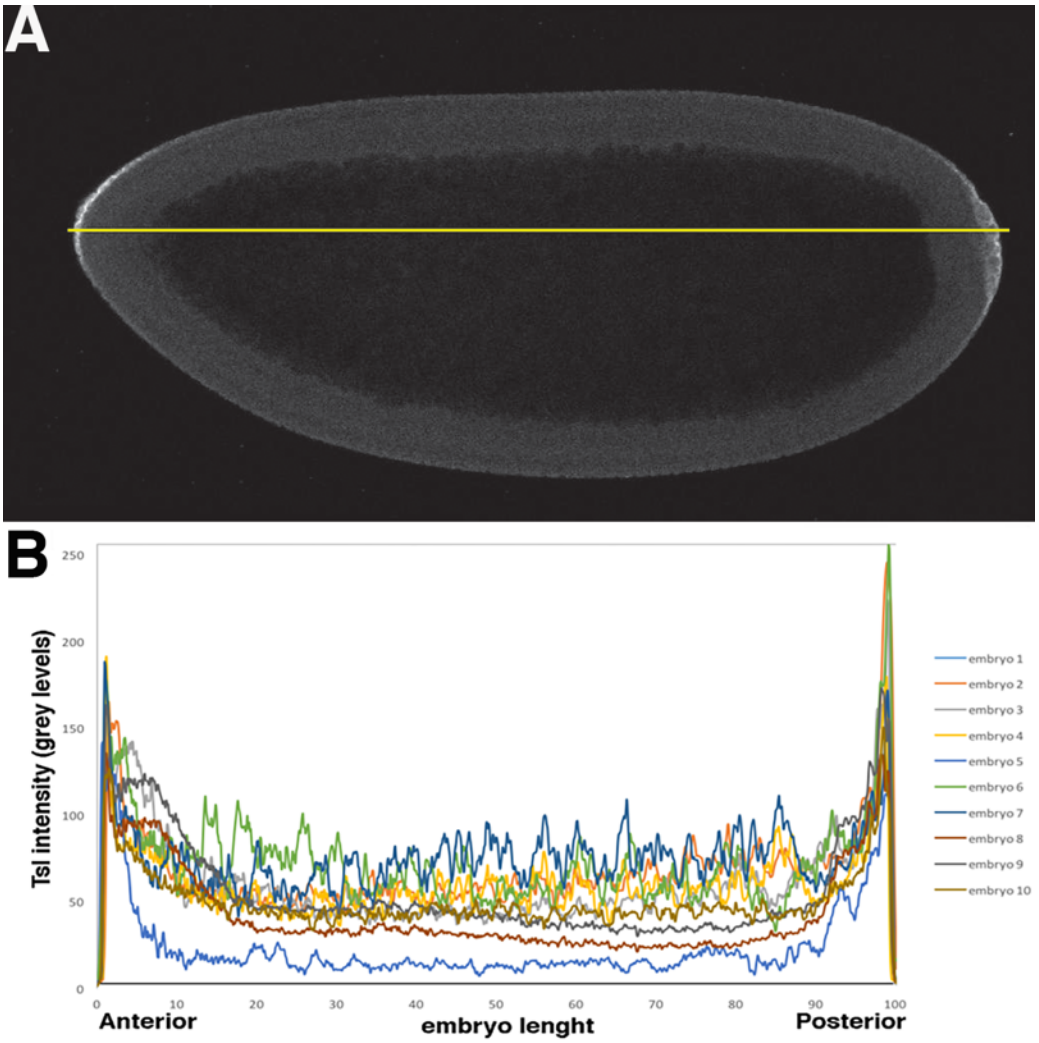

Figure.25 Quantification of the Tsl signal (A) Quantification of the Tsl signal from 10 embryos stained with the Tsl antibody. The line defines the embryo region used to measured the intensity of Tsl staining compared to the measure within the embryo as a control. (B)The instensity of the signal in grey intensity (from 0 to 256) is represented over the embryo length expressed in percentage (from 0 at the anterior pole to 100 at the posterior pole). The signal of Tsl is specifically localised at the poles.

Since we assessed the specificity of Tsl staining at the poles we then further analysed the requirements for Tsl accumulation. We analysed whether Tsl accumulation at the poles was dependent on the other genes involved in the terminal signalling. First we analysed whether it depended on the presence of the Tor receptor on the membrane surface. Indeed, since Tsl is the only component of the Tor signalling present at the poles, it was proposed that Tsl might act as the Tor ligand (Martin et al., 1994).

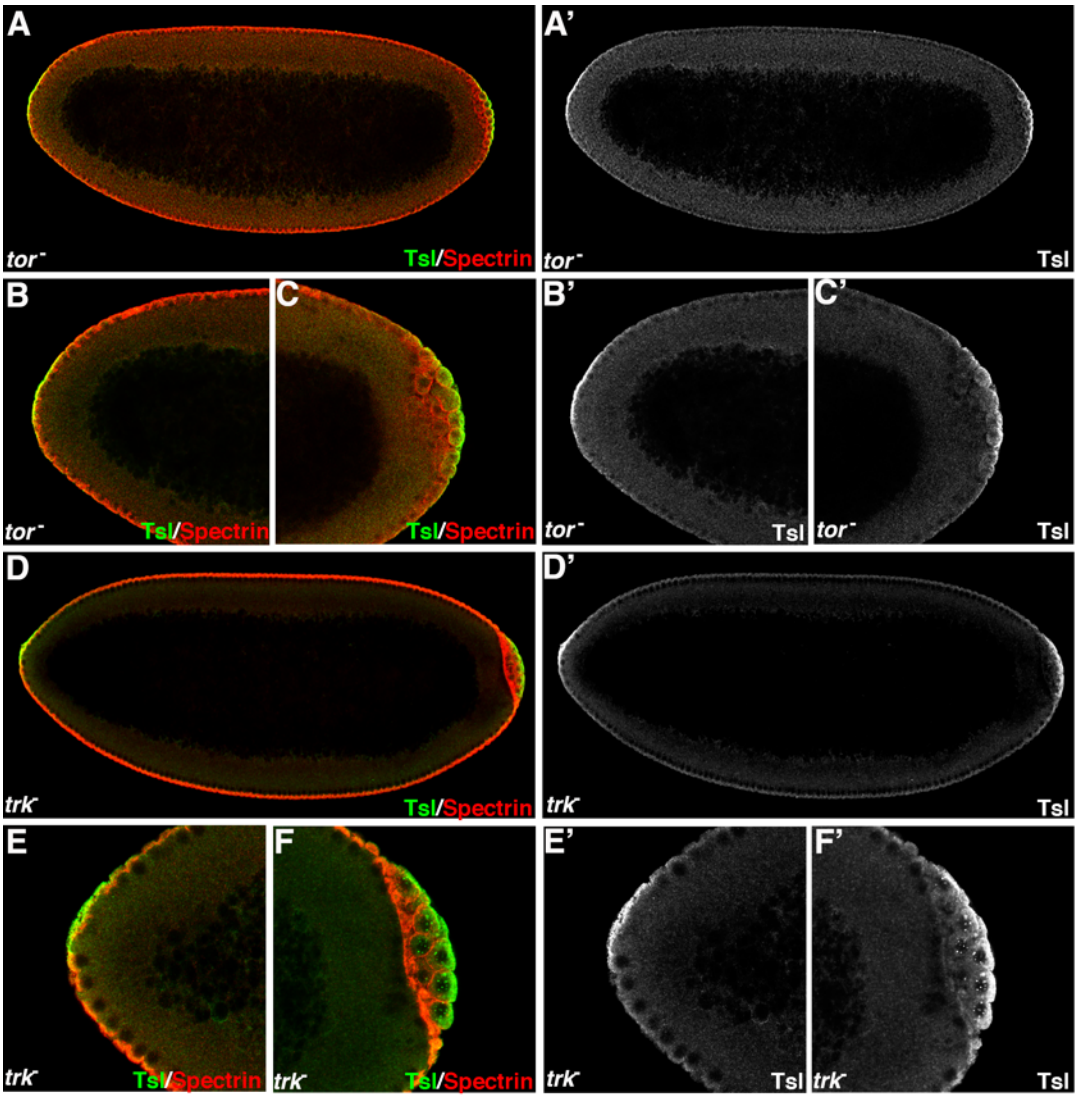

Figure.26. **Tsl accumulation independently of *tor* and *trk*.** (**A**) Projection of confocal sections of a syncytial blastoderm embryo laid by a *tor* null mutant female (tor^{XR}) stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. (**A'**) Same image in the green channel. (**B,C**) Magnifications of the same image including the anterior (B) and the posterior poles (C). (**B'-C'**) Same images in the green channel; antibody staining is detected at the poles of the embryonic plasma membrane. (**D**) Projection of medial

confocal sections of a syncytial blastoderm embryo laid by a *trk* null mutant female (trk^{RI53}) stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. (**D'**) Same image in the green channel. (**E,F**) Magnifications of the same image including the anterior (E) and the posterior poles (F). (**E'-F'**) Same images in the green channel; antibody staining is detected at the poles of the embryonic plasma membrane.

Although, different data have pointed to the protein encoded by the *trk* gene as the Tor ligand (Casanova et al., 1995), it remained an open question whether Tsl might be interacting with Tor at the embryo plasma membrane.

As shown in Fig.26A Tsl accumulates normally in embryos laid by *tor* (tor^{XT}) mutant mothers (these embryos are completely devoid of the Tor protein (Sprenger et al., 1989). This result suggests that Tsl accumulation at the embryo plasma membrane does not depend on the Tor receptor ruling out the possibility that Tsl is a Tor ligand and its localisation at the embryonic poles might reflect receptor binding.

Similarly, Tsl accumulates at the poles in embryos laid by *trk* mutant mothers. Therefore, Tsl accumulation does not depend either on the interaction with Trk, the putative Tor ligand (Fig.26D-F').

4.2 Tsl localization during oogenesis

Tsl has been described to accumulate at the poles in the inner surface of the embryonic vitelline membrane (Stevens et al., 2003). Since we unveiled that Tsl localised also at the embryonic plasma membrane, we speculated that Tsl translocates from the vitelline membrane to the embryonic plasma membrane. Indeed, as mentioned above, Tsl is a protein secreted in mid-oogenesis at stage 9 (Savant-Bhonsale and Montell, 1993), but does not performs its role until embryogenesis (Martin et al., 1994). Since in late oogenesis follicle cells in the egg chamber degenerate, a mechanism must exist to guarantee that the positional information, *i.e.* the restricted expression of *tsl* at the poles of the egg chamber, is maintained in early embryogenesis ensuring restricted activation of Tor receptor (Roth, 1998). We reasoned that such mechanism might consist in the anchorage of Tsl at the vitelline membrane upon its secretion during oogenesis followed by its translocation to the embryonic plasma membrane in embryogenesis. In this scenario, Tsl accumulation at the embryonic vitelline membrane (Stevens et al.,

2003) might indicate that not all of it it is translocated to the plasma membrane and, therefore, residual Tsl protein might be detected at the embryonic vitelline membrane. However, since Tsl extracellular accumulation upon secretion from the follicle cells is not well charachterised, so we examined where Tsl localisation using different markers. First, we found that Tsl accumulation overlaps with vitelline membrane markers such as Nasrat and Polehole (Fig.27A-B') (Ventura et al., 2010). This suggested that Tsl localised at the vitelline membrane. To discard that secreted Tsl does not localise at the oocyte plasma membrane, we used an antibody anti-Yolkless (Yl), the yolk receptor covering the oocyte plasma membrane (Schonbaum et al., 2000). Unfortunately, both Tsl antibody and Yl antibody are derived from rats making impossible to directly compare Tsl and Yl patterns. Therefore, since we found Tsl to overlap with Nasrat and Polehole, we examined the pattern of accumulation of Yl and compared with of Nasrat. We found that the two patterns did not overlap further corroborating that upon its secretion Tsl localised at the vitelline membrane (Fig.27C-C'). We also compared Tsl pattern with another structural component of the vitelline membrane such as VM32E (Andrenacci et al., 2001).

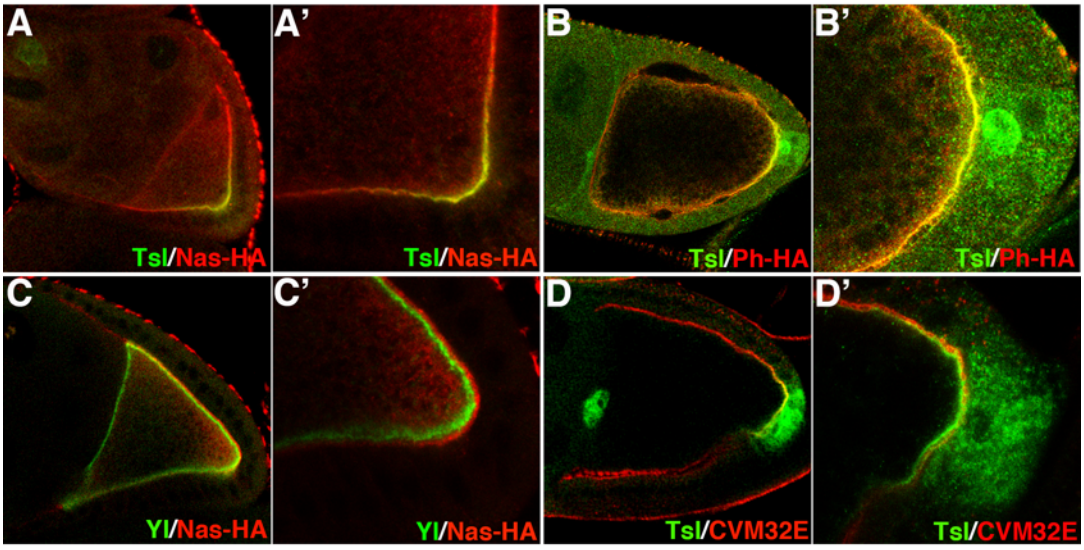

Figure.27 Tsl localisation in oogenesis. (**A**) Confocal section of a stage 9 egg chamber expressing the Nasrat-HA protein and stained with anti-Tsl (green) and anti-HA (red) antibodies. (**A'**) Magnification of the same egg chamber to show Tsl and Nasrat-HA co-localisation. (**B**) Confocal section of a stage 10 egg chamber expressing the Polehole-HA protein and stained with anti-Tsl (green) and anti-HA (red) antibodies. (**B'**) Magnification of the same egg chamber to show Tsl and Polehole-HA co-localisation.

(**C**) Confocal section of a stage 9 egg chamber expressing the Nasrat-HA protein and stained with anti-HA (green) and anti-Yl (red) antibodies. (**C'**) Magnification of the same egg chamber to show the different patterns of Nasrat-HA and Yolkless accumulation. (**D**) Confocal section of a stage 10 egg chamber stained with anti-Tsl (green) and anti-CVM32E (red) antibodies. (**D'**) Magnification of the same egg chamber to show the different patterns of Tsl and VM32E accumulation.

It has been shown that Polehole does not overlap with VM32E (Furriols and Casanova, 2014) and accordingly, we found that Tsl accumulation only partially overlaps with VM32E, and in many cases, they do not overlap being Tsl closer to the oocyte than VM32E (Fig.27 D-D'). Therefore, although Nasrat and VM32E have been found at the vitelline membrane by electron microscopy (Andrenacci et al., 2001; Ventura et al., 2010) and identified as components of the eggshell by proteomics (Fakhouri et al., 2006), they do not show overlapping patterns in confocal microscopy (Jimenez et al., 2002). This observation might indicate heterogeneity within the vitelline membrane suggesting that Tsl and Nasrat might be localised in the inner surface of the vitelline membrane which would fit with Tsl accumulation at the inner surface embryonic vitelline membrane (Stevens et al., 2003). The non-overlapping pattern of Nasrat and Tsl with VM32E might also be explained with differences in antibody accessibility or recognition caused by sample processing (chemical fixation or heat fixation). Indeed, since vitelline bodies are very dense structures, antibody accessibility inside them might be prevented, allowing their detection only at their periphery. This would explain that Nasrat and Polehole, secreted by the oocyte, are detected at the vitelline body periphery closer to the oocyte and, conversely, VM32E, being secreted from follicle cells, would be detected at the periphery of the vitelline bodies farther from the oocyte and closer to the follicular epithelium. Moreover, it has to be taken into account that VM32E has been identified as being also part of the endochorion (Andrenacci et al., 2001) while Nasrat has been identified as a component only of the vitelline membrane (Ventura et al., 2010). Although other endochorion components start to be secreted in late oogenesis at stage12, it might be that, even at earlier stages, VM32E is associated with the vitelline membrane but also with the nascent endochorion. If this were the case, it would explain why the Nasrat, Polehole and Tsl pattern only partially overlaps with VM32E.

In summary, these observations suggest that Tsl, upon its secretion in mid-oogenesis,

localise at the vitelline membrane probably through interactions with Nasrat, Polehole and Closca proteins (Jimenez et al., 2002; Stevens et al., 2003; Ventura et al., 2010). Therefore, as Tsl localise at the vitelline membrane during oogenesis and, later, at the plasma membrane in embryogenesis, Tsl has to translocate from the vitelline membrane to the embryo plasma membrane. Thus, we wondered what could be the trigger for Tsl translocation.

4.3 Tsl translocation occurs at egg activation

Tsl accumulation at the embryonic plasma membrane can be detected at very early stages of embryogenesis since the 3rd mitotic division (Fig.28 A-A').

As Tsl accumulation at the embryo poles occurs very early in embryogenesis we wondered whether it might be affected by fertilisation. We examined Tsl localisation in unfertilised oocytes and found that Tsl localised correctly, suggesting that Tsl localisation at the embryo poles is not linked to fertilisation (Fig.28 B-B'). Hence, we concluded that Tsl accumulates at the plasma membrane between late oogenesis and fertilisation. It is worth mentioning that in late oogenesis (stage11-14) protein detection within the egg chamber is prevented by the accumulation of the vitelline membrane and chorion proteins (Waring, 2000). Indeed, at stage 10B of oogenesis vitelline bodies fuse forming a continuos layer while chorion proteins start to be secreted at stage 12 by all follicle cells (Cavaliere et al., 2008). Thus, the eggshell completely covers the oocyte preventing antibody penetration into it.

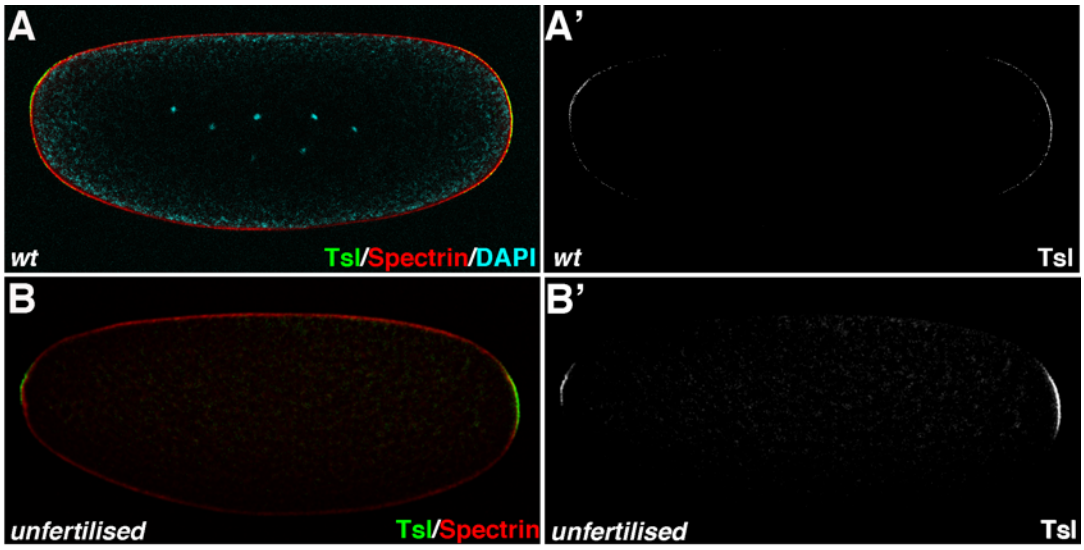

Figure.28 Tsl accumulation in early embryogenesis and unfertilised eggs. (**A**) Projection of confocal sections of an embryo in the 3rd mitotic division stained with anti-Tsl (green), anti-Spectrin (red) and DAPI (blue). (**A'**) Same image in the green channel. (**B**) Projection of confocal sections of an unfertilised egg laid by virgin females stained with anti-Tsl (green), anti-Spectrin (red). (**B'**) Same image in the green channel.

Due to this caveat, we could not analyse Tsl localisation in late oogenesis and, then, we cannot rule out the possibility that Tsl translocation might occur in late oogenesis. However, since no major changes in the vitelline membrane have been described to occur in these stages, Tsl translocation from the vitelline membrane to the plasma membrane is unlikely to occur in late oogenesis.

As oogenesis completes (stage 14), mature oocytes get funnelled through the oviduct to be deposited. In the oviduct, Ca^{2+} influx from the extracellular milieu converts the oocyte into an egg capable of supporting embryonic development undergoing lots of physiological changes that are defined as "egg activation".

The process of egg activation seems a very likely trigger for Tsl translocation for two main reasons. First, vitelline membrane non-disulfide cross linking is caused by egg activation and we have showed that Tsl, during oogenesis, anchors to the vitelline membrane (Fig.27). Therefore, it is tempting to speculate that the process of cross linking could affect, somehow, vitelline membrane proteins as Nasrat, Polehole and Closca modifying their interaction with Tsl, allowing its release in the perivitelline space and its translocation to the embryonic plasma membrane. Second, it has been

shown that when the oocyte passes through the oviduct, Ca^{2+} enters the oocyte in a wave starting from the poles (Kaneuchi et al., 2015). This is of particular interest because Perforin is strictly dependent on Ca^{2+} to bind to plasma membranes. Indeed, Ca^{2+} bind to Perforin negatively charged residues, triggering a conformational change that allows its interaction with the target plasma membrane (Voskoboinik et al., 2005). Therefore, an interesting possibility is that Ca^{2+} entry at egg activation induces a conformational modification in the Tsl protein allowing its interaction with the embryonic plasma membrane.

Unfortunately, a direct analysis of Tsl accumulation at the oocyte plasma membrane just before egg activation (stage 14) could not be performed. Indeed, as mentioned before, at this stage, the nascent chorion and vitelline membrane prevent antibody staining. To overcome this caveat, we tried to remove the eggshell either chemically (by a mixture of heptane and metanol 1:1) or manually. In both cases, we failed to obtain eggshell free occytes.

However, It has been shown that incubation of mature oocytes (stage 14) with a Ca^{2+} enriched buffer, "activation buffer" for 5 minutes reproduces all the events triggered by egg activation (Endow and Komma, 1997; Page and Orr-Weaver, 1997).

Therefore, we induced the process of egg activation *in vitro* and examined Tsl accumulation at the plasma membrane with the hope to isolate specific steps in Tsl translocation. In particular, we compared two different conditions. In the first one we took mature stage 14 oocytes and activated them *in vitro* for 5 minutes and, subsequently, heat-fixed and stained them with our Tsl antibody. Alternatively, mature stage 14 oocytes were activated *in vitro* for 5 minutes and left for 60 minutes in Zalokar buffer, a physiological buffer that allows embryonic development, prior to heat-fixation and antibody staining. We speculated that, comparing these conditions, we might be able to observe a progressive Tsl accumulation at the embryo plasma membrane. Therefore, we expected Tsl to be absent, or at least detected at very low levels, at the egg plasma membrane when oocytes were fixed immediately after egg activation. On the other hand, we speculated that allowing a "recovery" time after egg activation would allow the detection of Tsl at the plasma membrane, at least at higher levels compared to the previous condition.

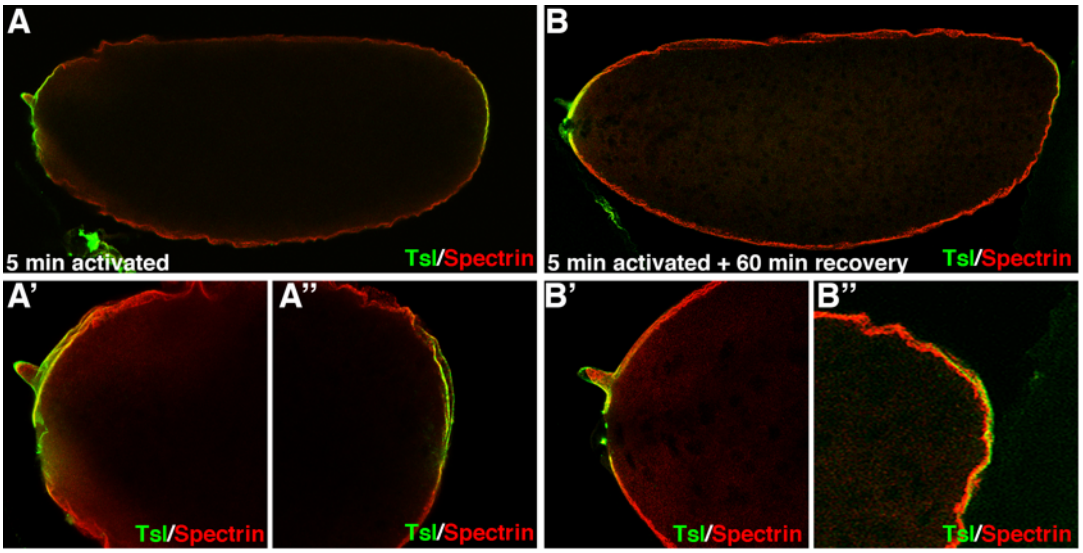

Figure.29 Tsl accumulation in vitro activated eggs (**A**) Stage 14 oocyte activated in vitro for 5 minutes. (**A'-A''**) Magnifications of the same image including the anterior (**A'**) and the posterior poles (**A''**). (**B**) Stage 14 oocyte activated in vitro for 5 minutes and left for 60 min in recovery buffer. (**B'-B''**) Magnifications of the same image including the anterior (**B'**) and the posterior poles (**B''**). As vitelline membrane and plasma membrane is tethered to the embryo plasma membrane so it is not possible to conclude whether Tsl translocates from the vitelline membrane to the embryo plasma membrane by effect of the in vitro egg activation process.

Unfortunately, we could not draw any conclusion from these experiments. In all the cases, as for the case of non-activated oocytes, we could not obtain eggshell free oocytes. Indeed, *in vitro* activated oocytes it was not possible to remove the vitelline membrane, either chemically or manually, because it was tethered to the embryo plasma membrane (an observation consistent with the ones of (LeMosy and Hashimoto, 2000).

As shown in Fig.29, in the oocytes from both conditions tested, it was not possible to conclude whether Tsl accumulates at the vitelline membrane or at the plasma membrane. Therefore, we decided to analise whether the process of egg activation triggers Tsl translocation using a genetic approach, examining oocytes in which the egg activation process is compromised.

It has been shown that the *sarah* gene encodes for calcipressin, a positive regulator of the calcium-dependent phosphatase calcineurin, that regulates the Ca^{2+} dependent cascade taking place at egg activation. At the moment of egg activation, Ca^{2+} enters

within the oocyte where it binds and activates calmodulin, which, in turn, activates downstream effectors such as calcineurin (Sartain and Wolfner, 2013).

Since the Sarah calcipressin is a calcineurin positive regulator, several aspects of egg activation are compromised in eggs laid by *sarah* mutant (Horner et al., 2006; Takeo et al., 2006). In particular, eggs laid by *sarah* (*sra*) mutant females arrest in anaphase of meiosis and fail to polyadenylate and translate bicoid mRNA but they undergo a correct crosslinking of the vitelline membrane (Fig.30 A) (Horner et al., 2006). Therefore, we analised whether Tsl translocation might be *sarah* dependent and found this not to be the case as Tsl accumulates properly at the plasma membrane in eggs laid by *sra* mutant females (Fig.30 B-B'). The localisation of Tsl in eggs laid by *sra* females suggests that Tsl translocation is not dependent on the intracellular signaling events occurring upon calcium influx at egg activation downstream of *sra* and, thus, that Tsl translocation is triggered by an egg activation event upstream of *sra.* Since the *sra* gene is one of the most upstream component of the signaling event that take place at egg activation, the likely trigger for Tsl translocation from the eggshell is either the calcium influx *per se* occurring at egg activation or the vitelline membrane crosslinking. Unfortunately, it is not possible to distinguish between these two possibilities. As mentioned above, the passage through the oviduct is supposed to induce the opening of a calcium channel allowing the entrance of Ca^{2+} from the external environment into the oocyte triggering egg activation (Sartain and Wolfner, 2013).

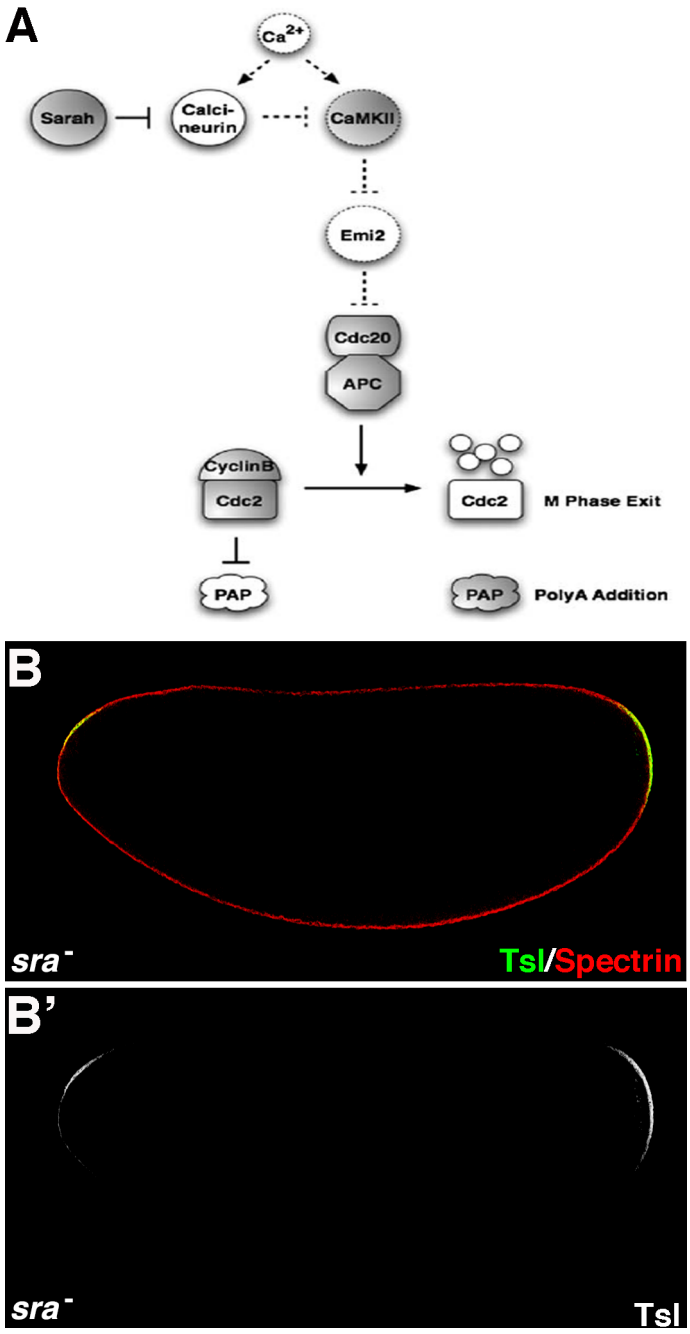

Figure.30 Tsl translocation is *sra* independent (A) The sarah calcipressin inhibits calcineurin allowing egg activation downstream events as cell cycle progression and maternal mRNAs polyadenylation (adapted from Horner et al., 2006). (**B**) Projection of confocal sections of an embryo laid by *sra* mutant females (sra^{A426}/sra^{KO}) stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. (**B'**) Same image in the green channel.

Since the molecular identity of this calcium channel remains elusive, it is impossible to prevent calcium influx occurring in the passage through the oviduct and, then, it is impossible to test whether Tsl translocation is dependent on Ca^{2+} influx.
Similarly, mutations affecting the vitelline membrane crosslinking, as the ones caused by *fs(N)*, *fs(ph)*, *clos* or *ndl*, give rise to oocytes that collapse upon egg laying and, hence, cannot be manipulated.

4.4 Tsl translocation is not affected by mutations in the MACPF domain

As mentioned above, it has been shown that negatively charged residues in the MACPF domain, in particular aspartate residues, play a crucial role for the insertion into the target cell plasma membrane (Voskoboinik et al., 2005). Aspartate residues are chelated by the Ca^{2+} in the external milieu inducing a conformational change that allows the association with the plasma membrane (Voskoboinik et al., 2005). Therefore, we wondered whether mutations in the MACPF domain could affect Tsl accumulation at the embryonic plasma membrane. Indeed, point mutations exist for the Tsl gene that abolish its function in Tor activation (Fig.30 (Savant-Bhonsale and Montell, 1993).

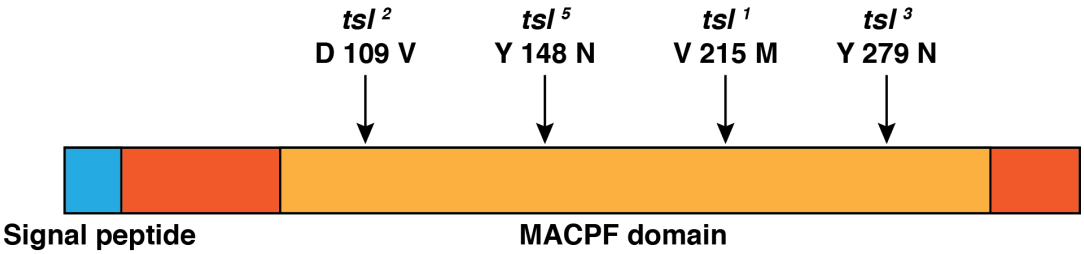

Figure.30 Overview of tsl point mutations. Schematic diagram of the Tsl protein with the amino acid substitutions found in the alleles of Tsl used in this work (adapted from Savant-Bhonsale and Montell, 1993).

Of note, the *tsl*^{2} allele is associated to a point mutation that causes an aminoacid change from an aspartate residue to a valine that might suggest that aspartate residues

in the Tsl protein play an important role as in the case of other MACPF proteins. For tsl^1, tsl^2, tsl^3 and tsl^5 point mutants, we found Tsl protein to accumulate at the embryonic plasma membrane (Fig.31), indicating that these mutations render the protein non-functional without interfering with its secretion, anchorage, or translocation to the embryonic plasma membrane.

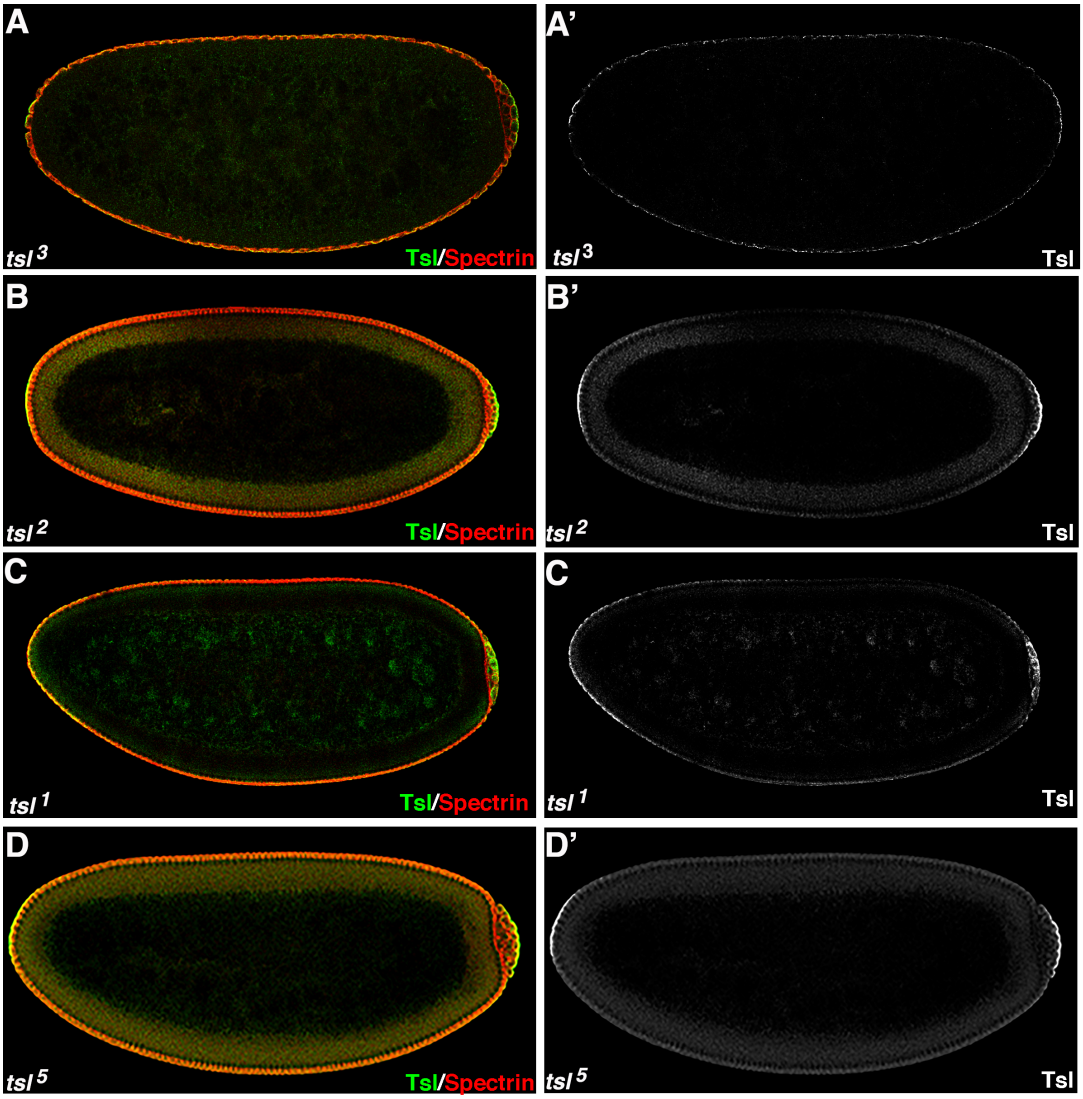

Figure.31 Tsl accumulation in *tsl* mutants. A) Projection of confocal sections of a syncytial blastoderm embryo laid by a tsl^3 mutant female (tsl^3/tsl^{604}) stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. **A'**) Same image in the green channel to show the specificity of the antibody staining. **B**) Projection of confocal sections of a syncytial blastoderm embryo laid by a tsl^2 mutant female (tsl^2 /tsl^{604}) stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. **B'**) Same image in the green channel to show the specificity of the antibody staining. **C**) Projection of confocal sections of a syncytial blastoderm embryo laid by a tsl^1 mutant female (tsl^1 /tsl^{604}) stained with anti-Tsl (green) and anti-

Spectrin (red) antibodies. **C'**) Same image in the green channel to show the specificity of the antibody staining. **D**) Projection of confocal sections of a syncytial blastoderm embryo laid by a tsl^5 mutant female (tsl^5 /tsl^{604}) stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. **D'**) Same image in the green channel to show the specificity of the antibody staining.

In particular, we observed a slight reduction in Tsl levels at the plasma membrane in tsl^3 compared to the control. However, tsl^3 does not appear to specifically affect translocation or accumulation of Tsl at the plasma membrane as it has been described that, in this case, the Tsl protein already fails to remain localised to the vitelline membrane (unpublished results in Stein et al., 2008).

4.5 Tsl localisation at the embryo plasma mebrane has a functional role for Tor activation

Since we have shown that Tsl translocates from the vitelline membrane to the embryonic plasma membrane at egg activation, we wondered whether Tsl performed its function at the embryonic plasma membrane or, as previously suggested, at the vitelline membrane (Stevens et al., 2003). To address this issue, we made use of a transgenic line previously generated in the laboratory expressing an amino-terminal HA-tagged Tsl protein (Tsl-HA) (Jimenez et al., 2002). It was reported that this HA-tagged Tsl protein was able to provide *tsl* function in regulating developmental timing but not in terminal patterning (Johnson et al., 2013). Therefore, we wondered whether the lack of activity in terminal patterning of Tsl-HA was due to a lack of accumulation at the plasma membrane suggesting an improper translocation from the vitelline membrane.

We examined Tsl-HA localisation in the egg chamber and found that it localised at the vitelline membrane as its pattern of accumulation overlapped with a RFP-tagged form of Vitelline Membrane Like, a vitelline membrane protein (Zhang et al., 2009) (Fig.32A-A'). Then, we analised Tsl-HA accumulation in the embryo and found that it was absent, or at least not detectable, in the embryonic plasma membrane (Fig.32 E-E'). To exclude that this was due to a failure of the anti-HA antibody in recognising Tsl-HA at the plasma membrane, we used the anti-Tsl antibody in flies devoid of endogenous Tsl but carrying the Tsl-HA transgene. In this case, we were able to detect Tsl-HA in the egg chamber (Fig.32C-C') but, nevertheless, we could not detect Tsl-HA

in the embryonic plasma membrane (Fig.32F-F'). As a control for the Tsl dosis, we confirmed that we could detect Tsl accumulation at the embryo poles even in a tsl hemyzigous background (Fig.32 G-G').

These results suggest that Tsl accumulation at the embryonic plasma membrane has a role for terminal patterning. Indeed, this result suggests that Tsl-HA, which retains Tsl activity in developmental timing (Johnson et al., 2013), is not functional in the context of embryonic terminal patterning due to its lack of translocation from the eggshell to the embryonic plasma membrane.

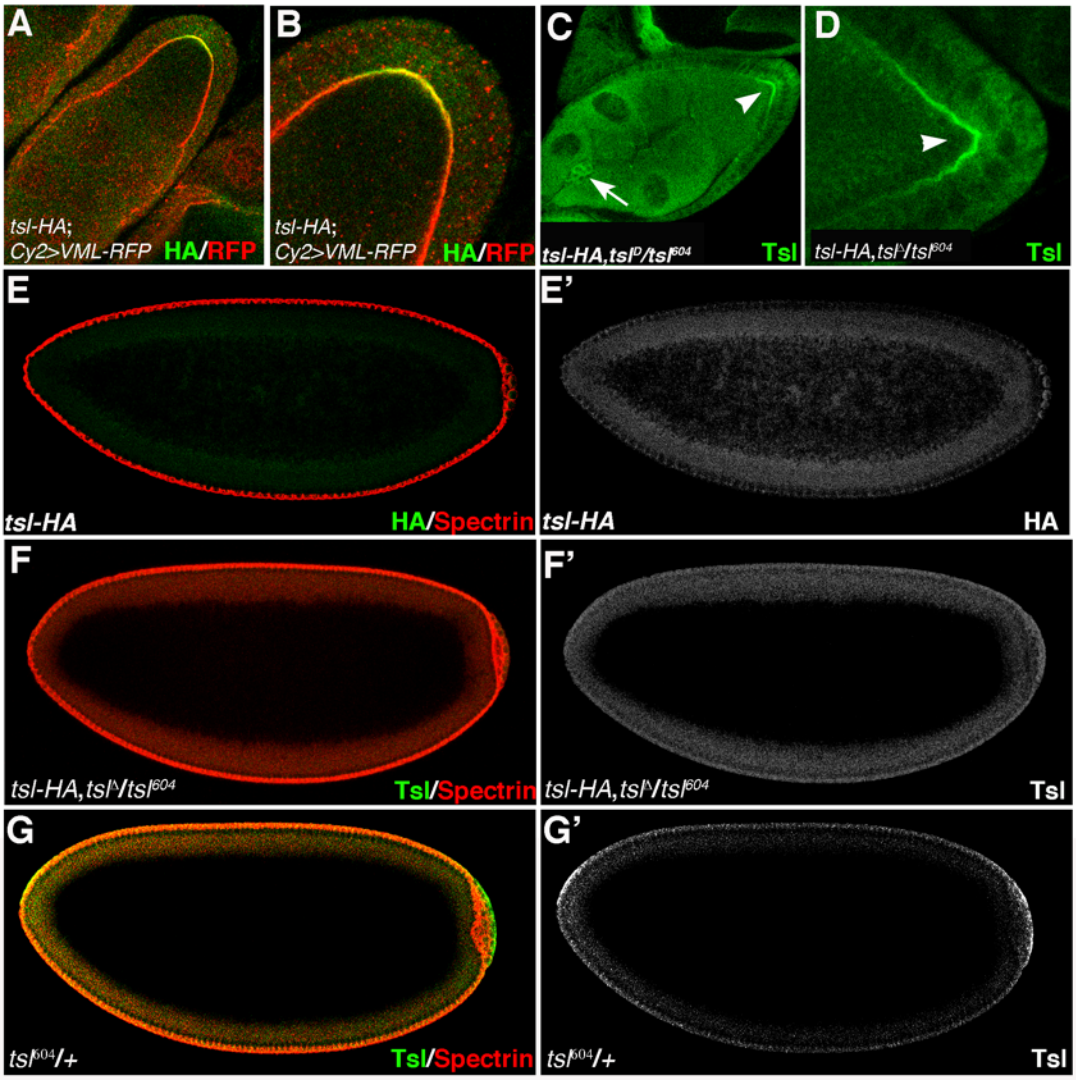

Figure.32 Tsl-HA does not translocate to the embryonic plama membrane. A) Confocal section of a stage 9 egg chamber expressing the Tsl-HA protein and the RFP tagged Vitelline membrane-like (VML) (Zhang et al., 2009a) protein under the control of the Cy2-Gal4 driver and stained with with anti-HA

(green) and anti-RFP (red) antibodies. **B**) Magnification of the same egg chamber to show Tsl-HA and VML-RFP co-localisation in the vitelline membrane. **C**) Confocal section of a stage 9 egg chamber expressing the Tsl-HA protein in a tsl^{Δ}/tsl^{604} mutant background and stained with anti-Tsl (green) antibody to show accumulation of Tsl-HA in the border cells (arrow) and extracellular accumulation in the posterior pole secreted by the posterior follicle cells (arrowhead). **D**) Confocal section of a stage 9 egg chamber expressing the Tsl-HA protein in a tsl^{Δ}/tsl^{604} mutant background and stained with anti-Tsl (green) antibody to show in more detail the extracellular accumulation of Tsl-HA in the posterior pole (arrowhead). **E**) Projection of medial confocal sections of a syncytial blastoderm embryo expressing the Tsl-HA protein and stained with anti-HA (green) and anti-Spectrin (red) antibodies. **E'**) Same image in the green channel to show the lack of detection of the Tsl-HA protein with the HA antibody (3F10). **F**) Projection of medial confocal sections of a syncytial blastoderm embryo expressing the Tsl-HA protein in a tsl^{Δ}/tsl^{604} mutant background and stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. **F'**) Same image in the green channel to show the lack of detection of the Tsl-HA protein with the Tsl antibody.

Since in the Tsl-HA construct the HA tag was inserted at the amino terminal part of the Tsl protein (outside of the MACP domain) (Jimenez et al., 2002), lack of localisation of Tsl-HA at the embryo plasma membrane might suggest that the amino terminal part of the Tsl protein is important for Tsl translocation but it is dispensable for Tsl function in regulating developmental timing (Johnson et al., 2013).

4.6 Tsl translocation does not depend on the Nasrat, Polehole and Clos

As mentioned in the introduction, Nasrat, Polehole and Closca are required for Tsl anchorage/stabilisation in oogenesis as secreted Tsl is not detected in the egg chambers of *fs(1)N, fs(1)ph* and *clos* null females (Jimenez et al., 2002; Ventura et al., 2010). In the embryo, very much reduced Tsl levels were detected in the inner side of the vitelline membranes of embryo laid by $fs(1)N^{12}$ and $fs(1)ph^{1901}$ flies corroborating the role of Nasrat, Polehole in stabilising/anchoring the Tsl protein at the vitelline membrane (Stevens et al., 2003). Nevertheless, upon ectopic activation in all follicle cells in egg chambers from $fs(1)N^{12}$ and $fs(1)ph^{1901}$ mothers, high Tsl levels can be detected at the vitelline membranes of embryos laid by these mothers even though it does not rescue the lack of terminal structures (Stevens et al., 2003). These data indicated that Nasrat, Polehole and Closca had an additional role in terminal patterning

besides stabilising/localising Tsl. Thus, it was suggested that Nasrat and Polehole were also required for "Tsl to exert its function" (LeMosy, 2003; Stevens et al., 2003).

Thus, we examined whether this additional role could indeed be related to the translocation of Tsl from the vitelline membrane to the embryonic plasma membrane. We first analysed Tsl accumulation in embryos laid by by *fs(1)N*^{12} and *fs(1)ph*^{1901} mutant females (Fig.33 A-B') and found Tsl to be absent or at least not detectable in the plasma membrane of these embryos.

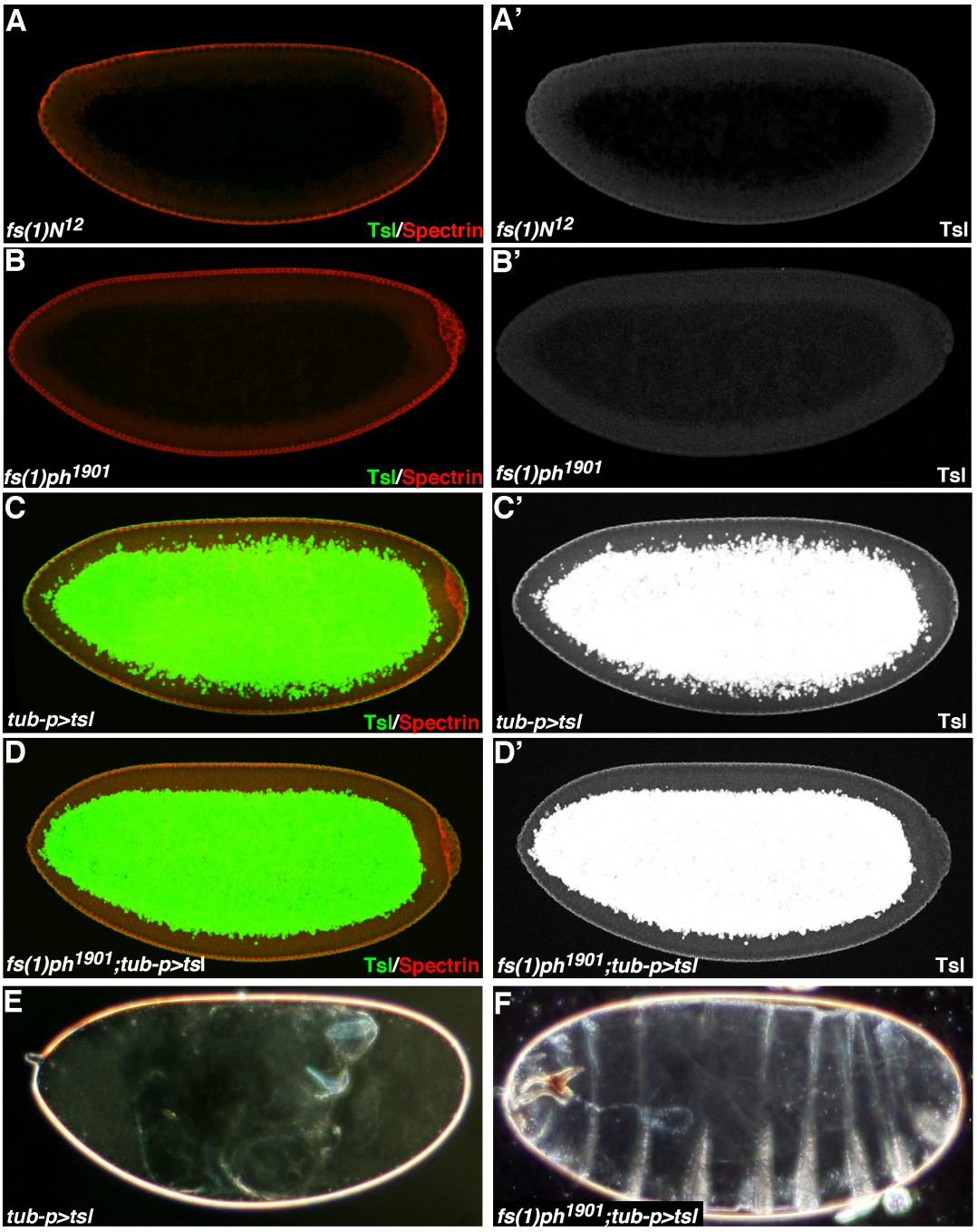

Figure.33 *fs(1)N*^{12} and *fs(1)ph*^{1901} do not affect Tsl translocation A) Projection of confocal sections of a syncitial blastoderm embryo laid by a *fs(1)Nas*^{12} stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. **(A')** Same picture showing only Tsl accumulation; Tsl is not detected at the embryonic poles of *fs(1)N*^{12} terminal mutants. **(B)** Projection of medial confocal sections of a syncitial blastoderm embryo laid by a *fs(1)ph*^{1901} terminal mutant female stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. **(B')** Same picture showing only Tsl accumulation; Tsl is not detected at the embryonic poles of *fs(1)ph*^{1901} terminal mutants. **(C)** Projection of medial confocal sections of a syncitial blastoderm embryo laid by a *fs(1)ph*^{1901}/+ female overexpressing Tsl in all follicle during oogenesis with the

Tubulin-Gal4 driver and stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. **(C')** Same picture showing only Tsl accumulation; Tsl is detected ectopically all around the embryonic plasma membrane. **(D)** Projection of medial confocal sections of a syncitial blastoderm embryo laid by a *fs(1)ph*^{1901} mutant female overexpressing Tsl in all follicle during oogenesis with the Tubulin-Gal4 driver and stained with anti-Tsl (green) and anti-Spectrin (red) antibodies. **(D')** Same picture showing only Tsl accumulation; Tsl is detected ectopically associated with the embryonic plasma membrane even in a *fs(1)ph*^{1901} mutant background. (**E**) Cuticle of an embryo laid by a *fs(1)ph*^{1901}/+ female overexpressing Tsl in all follicle cells. Overexpression of Tsl give rise to a spliced phenotype where terminal structures expand at the expenses of the others. (**F**) Cuticle of an embryo laid by a *fs(1)ph*^{190} female overexpressing Tsl in all follicle cells. The embryo has a terminal phenotype.

However, since Tsl is also very much reduced in the vitelline membrane of the eggs laid by *fs(1)N*^{12} and *fs(1)ph*^{1901} mutant females, it might also be the case that not enough Tsl protein is available to translocate to the embryonic plasma membrane. To assess whether the lack of Tsl accumulation was due to a failure in the translocation process or a reduction in its levels at the vitelline membrane, we ectopically expressed *tsl* in all follicle cells of *fs(1)ph*^{1901} mutant females and found Tsl to accumulate in the embryonic plasma membrane (and even within the embryos) (Fig. 33 C-D') as it is also the case in the vitelline membranes of embryos laid by the same mutant females (Stevens et al., 2003).

These results show that Nasrat/Polehole/Closca are required in the anchorage of Tsl to the eggshell and not in its succeeding translocation to the embryo. Lack of terminal activity observed upon ectopic expression of Tsl in embryos laid by *fs(1)ph*^{1901} females (Fig. 33 E-F) could account for a failure in the proper anchorage of Tsl to the plasmatic membrane suggesting that Nasrat, Polehole and Closca could mediate an additional role on Tsl making it functional as already suggested (Stevens et al., 2003). Alternatively, it might be that Nasrat, Polehole and Closca play a role on another component of the terminal system independently of the anchorage of Tsl at the eggshell.

4.7 A new Tsl independent function for Nasrat, Polehole and Closca in terminal system

Since we found that Nasrat, Polehole and Closca are not involved in the translocation of Tsl from the vitelline membrane to the embryonic plasma membrane, we wondered

whether the additional role of Nasrat, Polehole and Clos in terminal patterning could indeed be Tsl independent. As already described in the introduction, Tor activation at the embryonic poles by its ligand Trk is mediated by the Tsl protein in a still unknown mechanism (Furriols and Casanova, 2003). It also has been shown that, upon its ectopic expression in the germline, PTTH can activate Tor ectopically in a Tsl independent manner (Johnson et al., 2013). Therefore, we wondered whether PTTH might be dependent on Nasrat, Polehole and Closca to activate Tor. We found that, while ectopic PTTH expression in the germline activates Tor even in the absence of Tsl (Fig.34A), it requires Polehole to induce the formation of terminal structures (Fig.34B). This result indicates that Nasrat, Polehole and Closca are necessary for proper Tor activation and suggest that they might have a function in the terminal system that is independent of Tsl. To exclude that overexpression in the germline does not produce enough PTTH levels to activate Tor in embryos laid by *fs(1)ph*^{1901} mutant mothers, we misexpressed PTTH in all follicle cells using the α-tubulin-Gal4 (tub-p-Gal4) driver.

We found that, in this condition, PTTH is still active and activates Tor at very high levels giving rise to a strong spliced phenotype (Fig.34 C). Nevertheless, high PTTH levels are not sufficient to induce Tor activation in in embryos laid by *fs(1)ph*^{1901} mutant mothers (Fig.34 D).

These data indicate that while Nasrat, Polehole and Closca are not required for PTTH mediated Tor activation in the Prothoracic Gland (Grillo et al., 2012), they are necessary for Trk-mediated Tor activation. As ectopic expression of PTTH in the embryo activates Tor ectopically even in the absence of Tsl but requires Polehole, these results suggest that Nasrat, Polehole and Closca might have an additional Tsl-independent function on the terminal system.

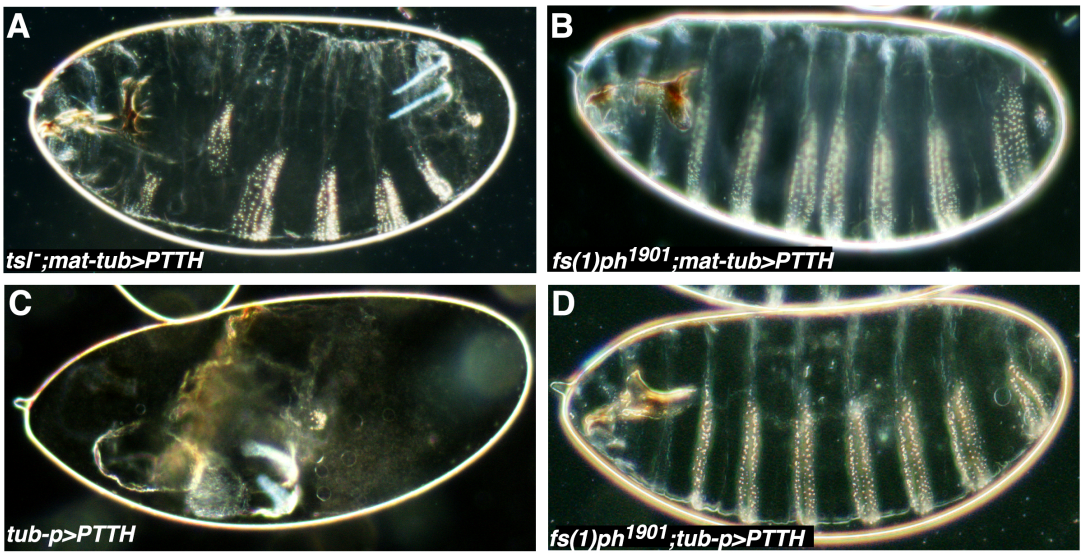

Figure.34 PTTH overexpression rescues *tsl* but not *fs(1)ph^{1901}* **A**) Cuticle of a embryo from *tsl^{604}/Def (3R)$^{cakiX-313}$* female overexpressing Ptth in the germline (mat-tub>PTTH). PTTH overexpresion rescues the lack the terminal structures of *tsl* mutant embryos and ectopically activates Tor signalling (Johnson2013). **B**) Cuticle of an embryo from *fs(1)ph^{1901}* mutant female overexpressing PTTH in the germline (mat-tub>PTTH). All the embryos laid by these females have a terminal phenotype lacking all terminal structures. **C**) Cuticle of an embryo overexpressing Ptth in all follicle cells (tub-p>PTTH). PTTH overexpression in all follicle cells strongly activates Tor signalling giving rise to a "spliced" phenotype where terminal structures expand at the expenses of the other structures **D**) Cuticle of an embryo from *fs(1)ph^{1901}* mutant female overexpressing PTTH in in all follicle cells (tub-p>PTTH). All the embryos laid by these females have a terminal phenotype lacking all terminal structures.

5. Nasrat, Polehole and Closca act as common mediators of dorso-ventral and terminal patterning

5.1 Ndl localization in the egg chamber

Nasrat, Polehole and Closca proteins are secreted by the oocyte in mid oogenesis (stage7-8) and accumulate in the vitelline membrane (Grillo et al., 2012; Jimenez et al., 2002; Ventura et al., 2010). On the other hand, the Ndl protein is secreted during mid-oogenesis (stage7-8) by the follicle cells (Hong and Hashimoto, 1995), and it has been showed to accumulate at the oocyte plasma membrane (LeMosy et al., 1998) although it has also been identified as a component of the eggshell by proteomics (Fakhouri et al 2006). Therefore, we examined Ndl accumulation in the egg chamber using different markers. We found that secreted Ndl distribution overlaps with that of a vitelline membrane component as Polehole (Fig.35A-A') or Tsl (Fig.35C-C') and does not overlap with the the yolk receptor Yolkless (Yl) that covers the oocyte plasma membrane (Fig.35B-B') (Schonbaum et al., 2000). These observations suggested that, during oogenesis, Ndl localise at the vitelline membrane. As already mentioned in the introduction, Ndl was previously reported to accumulate at the embryonic plasma membrane in embryogenesis (LeMosy et al., 1998). The observation that Ndl localise at the vitelline membrane in oogenesis, rather than the oocyte plasma membrane, suggests that Ndl might be translocated from the vitelline membrane to the plasma membrane at early embryogenesis.

5.2 Proper Ndl localisation in the egg chamber depends on Nasrat, Polehole and Closca

Since we found that Ndl localise at the vitelline membrane col-localising with Polehole, we wondered whether Nasrat, Polehole and Closca might be required for Ndl localisation. We found that, in stage 9 egg chambers from null *fs(1)ph* mutant females, which, as mentioned above, also lack Nasrat and Clos accumulation, Ndl is partially mislocalised as it can be also detected within the oocyte (Fig.36A-B').

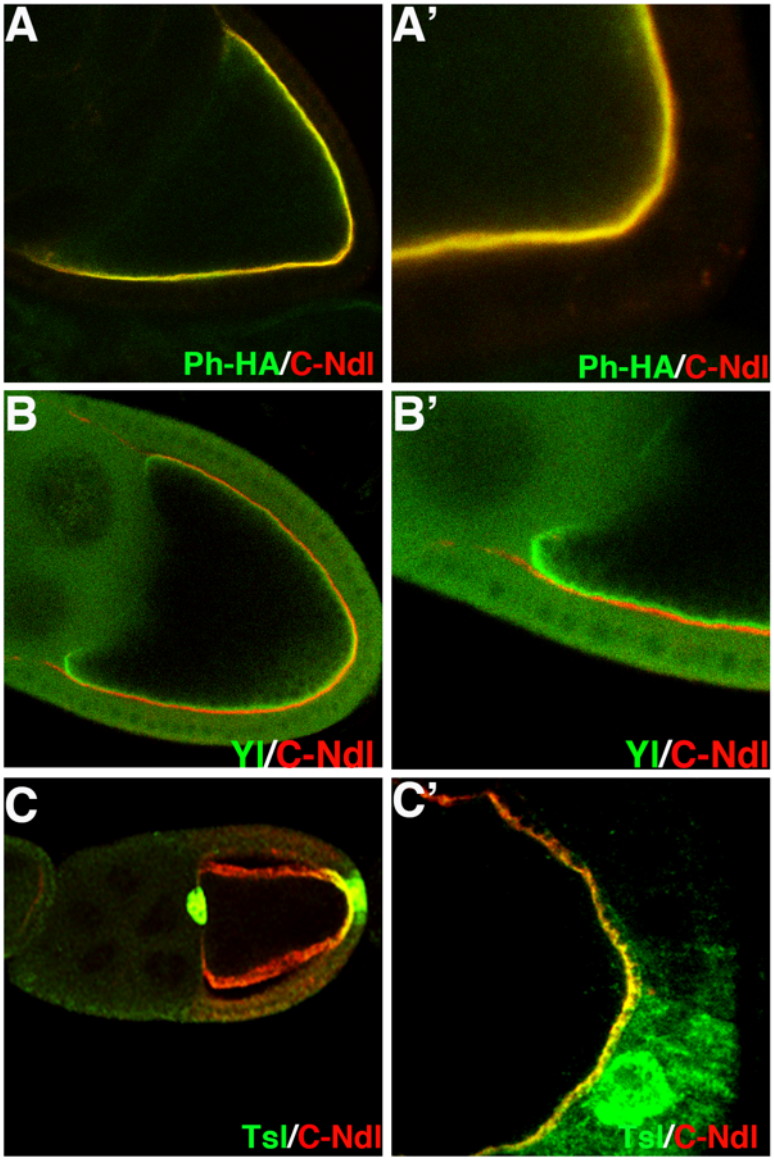

Figure.35 During oogenesis Ndl localise at the vitelline membrane A) Confocal section of a stage 9 egg chamber expressing Polehole-HA and immunostained with anti-C-Ndl (in red) and anti-HA (in green) antibodies. **A′**) A magnification of A showing the colocalisation. **B**) Confocal section of a stage 9 egg chamber immunostained with anti-C-Ndl (in red) and anti-Yl (in green) antibodies. **B′**) A magnification of B showing the lack of colocalisation. **C**) Confocal section of a stage 10 egg chamber expressing Tsl and immunostained with anti-C-Ndl (in red) and anti-Tsl (in green) antibodies. **C′**) A magnification of A showing the colocalisation.

This result suggests that Ndl requires Nasrat, Polehole and Clos to be properly anchored or stabilised at the vitelline membrane. However, by stage 10 of oogenesis, we did not detect Ndl inside the oocyte suggesting that the non-localised Ndl protein gets degraded and/or eliminated by this stage (Fig.36C-D'). At stage 12-14 we also

detected an abnormal pattern. In *wild type* egg chambers Ndl accumulates in punctae between the oocyte plasma membrane and the follicle cells as described for other vitelline membrane proteins (Furriols and Casanova, 2014a). Conversely, in approximately 70% of the egg chambers from *fs(1)ph* mutant females, Ndl shows a lumpy distribution between the oocyte plasma membrane and the follicle cells (Fig.36E-F'). We confirmed that Ndl pattern showed similar defects in egg chambers from *fs(1)N* and *clos* mutant flies(Fig.37).

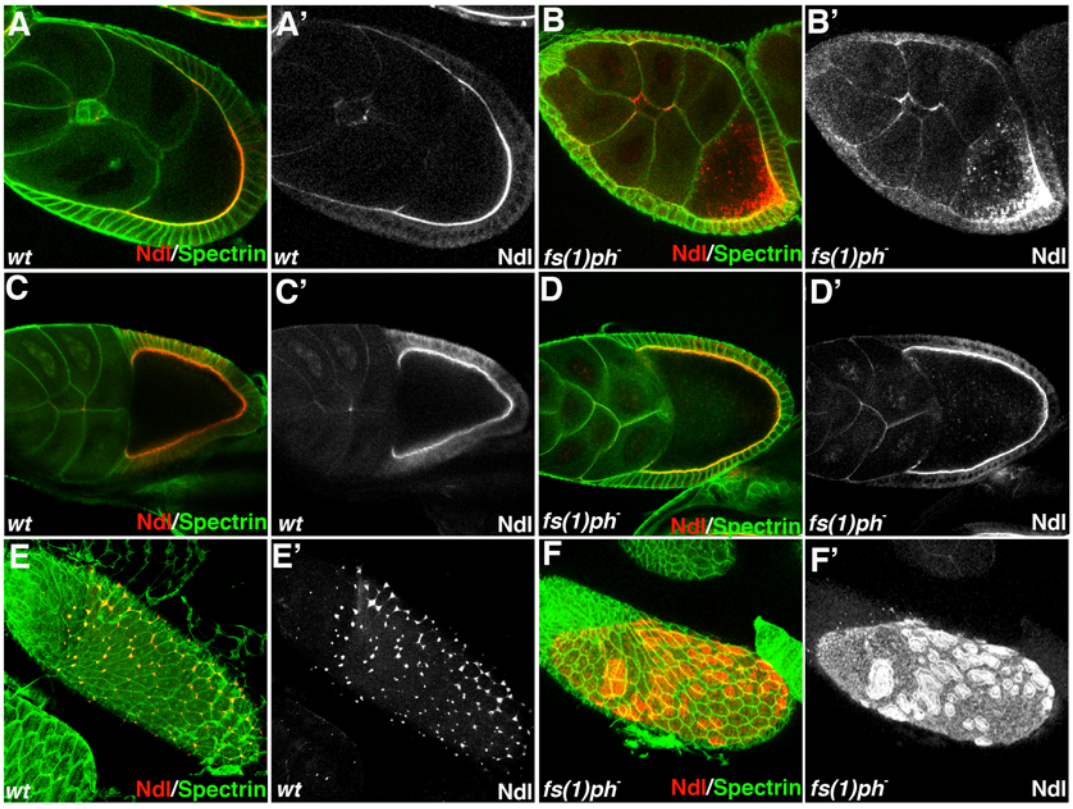

Figure.36 Polehole is required for Ndl achorage or stabilisation in oogenesis. A-F) Confocal sections of *wt* and *fs(1)ph*^{K646} egg chambers at different stages immunostained with anti-C-Ndl (in red) and anti-Spectrin (in green) antibodies. (A,B) Stage 9; (C,D) Stage 10; (E-F) stage 12. **A′-F**′) Same images in the red channel. Note mislocalised Ndl inside the oocyte at stage 9 (B,B′) and the Ndl lumpy distribution at stage 12 (F-F′) in *fs(1)ph*^{K646} egg chambers .

Since we found that Ndl anchorage or stabilisation at the vitelline membrane partially depends on Nasrat, Polehole and Closca we wondered whether this was due to a specific role of these proteins on Ndl. To address this issue, we examined whether the structural vitelline membrane components were affected in egg chambers from *fs(1)N*

mutant mothers and found this not to be the case as sV23 and VM32E localise correctly (Fig.38A-B). While Nasrat, Polehole and Clos were found to be required for Ndl localisation, the opposite was not true, as Polehole and also Tsl localised correctly in egg chambers from *ndl* null flies (Fig.38 C-D).

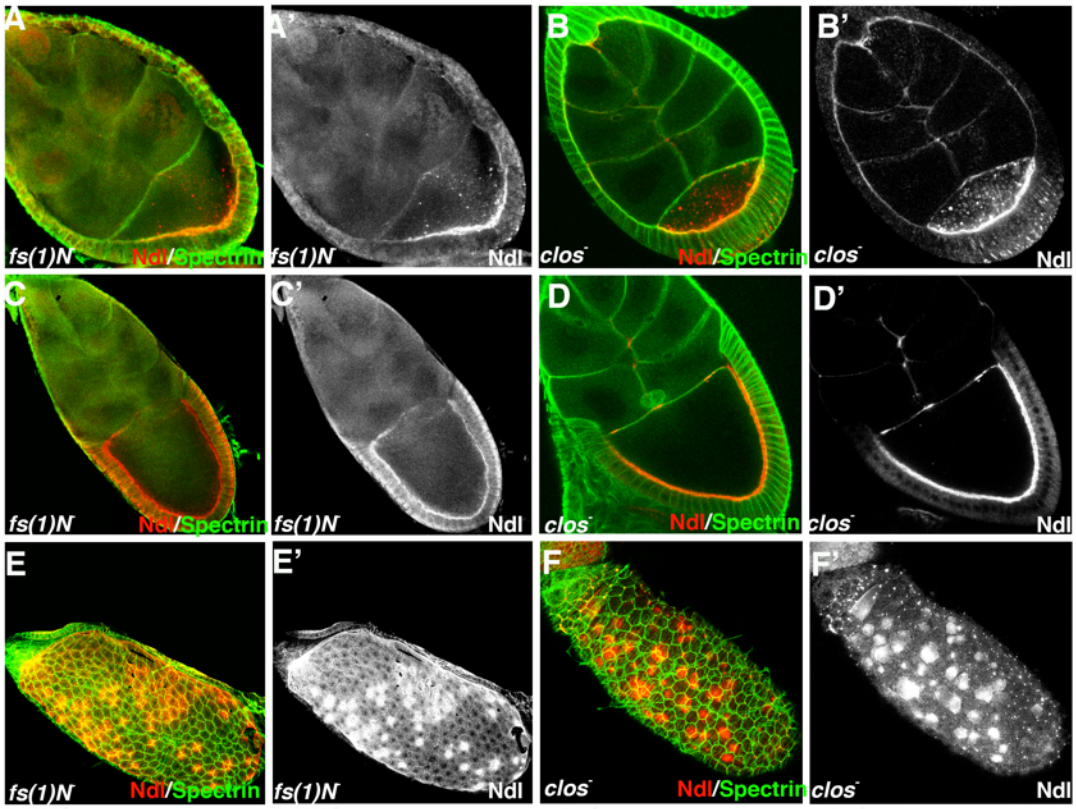

Figure.37 Nasrat and Closca are required for Ndl achorage or stabilisation in oogenesis. (**A-F**) Confocal sections of *fs(1)N*^{14} and *clos*^{4152} egg chambers at different stages immunostained with anti-C-Ndl (in red) and anti-Spectrin (in green) antibodies. (A,B) Stage 9; (C,D) Stage 10; (E-F) stage 12. (**A′-F′**) Same images in the red channel. Note mislocalised Ndl inside the oocyte at stage 9 (A-B′) and the Ndl lumpy distribution at stage 12 (E-F′).

To confirm that Ndl mislocalisation was due to a specific role of Nasrat, Polehole and Closca on Ndl, we analised whether Ndl localisation might be dependent on other structural components of the vitelline membrane. Therefore, we examined Ndl localisation in egg chambers in from *Vm26Ab*^{QJ42} mutant mothers, which completely lack the sV23 protein (Savant and Waring, 1989), and found that Ndl as well as Polehole or Tsl localise correctly(Fig.38E-G). It has been shown that proper vitelline membrane structure requires components of the vitelline membrane, as sV23 (Savant

and Waring, 1989), or proper structure of the microvilli. Indeed, mutations in sV23, Palisade and in Caderin99C that are required for proper microvilli organisation (Elalayli et al,. 2008, Schlichting et al., 2006), alter the structure or the localisation of vitelline membrane components respectively. In contrast, our observations suggest that Nasrat, Polehole and Clos do not alter the composition of the vitelline membrane either but have a specific role on Ndl.

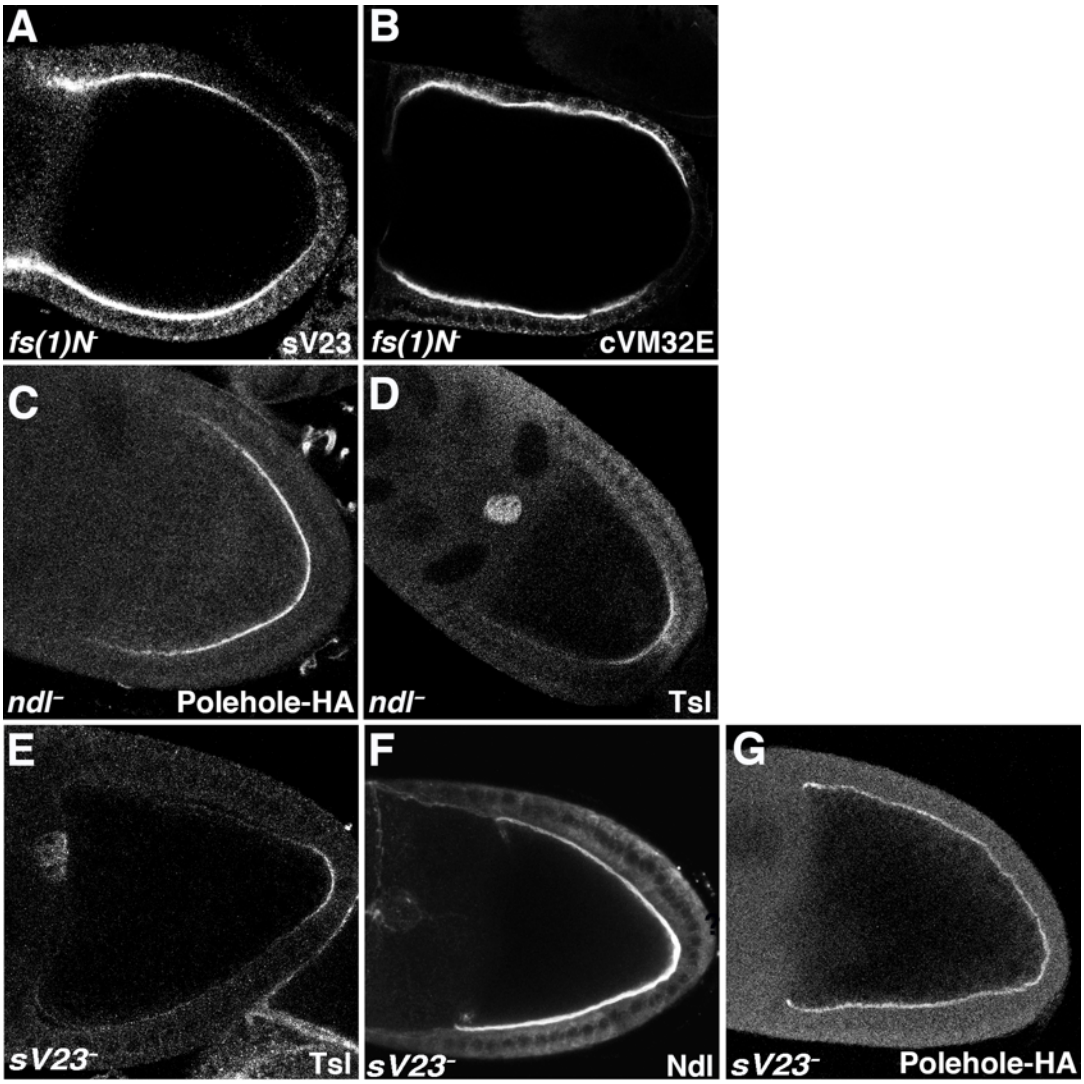

Figure.38 (**A**) Confocal section of a Nasrat null (*fs(1)N*^{14}) stage 9 egg chamber immunostained with anti-sV23 antibody. (**B**) Confocal section of a Nasrat null (*fs(1)N*^{14}) stage 10 egg chamber immunostained with anti-cVM32E antibody. Note the absence of VM32E from the posterior follicle cells (andrenacci 2001). (**C**) Confocal section of a *ndl* null (*ndl*^{14}*/Def(3L)CH12)* stage 9 egg chamber expressing the Polehole-HA and immunostained with anti-HA antibody. (**D**) Confocal section of a *ndl* null (*ndl*^{14}*/Def(3L)CH12)* stage 9 egg chamber immunostained with anti-Tsl antibody. (**E**) Confocal

section of a sV23 null (*Vm26Ab*^{QJ42}) stage 9 egg chamber immunostained with anti-Tsl antibody. (**F**) Confocal section of a sV23 null (*Vm26Ab*^{QJ42}) stage 9 egg chamber immunostained with anti-C-Ndl antibody.(**G**) Confocal section of a sV23 null (*Vm26Ab*^{QJ42}) stage 9 egg chamber expressing the Polehole-HA and immunostained with anti-HA antibody.

5.3 Role of *fs(1)N*^{12}, *fs(1)ph*^{1901} and *clos*^{1} in dorsoventral patterning and eggshell integrity

As already mentioned in the introduction, the Ndl protease is necessary for vitelline membrane crosslinking and for dorso-ventral axis specification. Since *fs(1)N*, *fs(1)Ph* and *clos* are also necessary for vitelline membrane crosslinking and for proper Ndl anchorage or stabilisation at the vitelline membrane, we wondered whether these proteins might also be required for dorso-ventral patterning. Unfortunately, oocytes laid by *fs(1)N*, *fs(1)Ph* and *clos* null flies collapse upon treatment with bleach or do not complete embryonic development (Degelmann et al., 1990; Jimenez et al., 2002; Ventura et al., 2010). Therefore, we could not study the patterning defects in the progeny from these mutant females.

Thus, we wondered whether embryos laid by *fs(1)N*^{12}, *fs(1)ph*^{1901} and *clos*^{1} alleles might display dorso-ventral defects. Although, no defects in dorso-ventral patterning were previosuly observed in these embryos (Degelmann et al., 1990; Jimenez et al., 2002), we extended this analysis by examining them at different temperatures. We found that, when these mothers were raised at 18ºC, they displayed their terminal phenotype although, when incubated at 25ºC or, in particular, at 29ºC, these flies laid embryos that, besides the loss of terminal structures, had a dorsalised phenotype (Fig.39 and Table 1). We also observed differences in the percentage of dorsalised embryos in the progeny of *fs(1)N*^{12}, *fs(1)ph*^{1901} and *clos*^{1}. In particular, *clos*^{1} females (*clos*^{1}/*clos*^{4152}) laid more embryos showing a dorsalised phenotype compared to *fs(1)N*^{12} and *fs(1)ph*^{1901} homozygous females.

	***fs(1)N*^{12}**	***fs(1)ph*^{1901}**	***clos*^{1}/*clos*^{4152}**	***fs(1)ph*^{1901}; Ph-HA/+**
18ºC	**1%**	**3%**	**1%**	**/**
25ºC	**1%**	**8%**	**7%**	**/**
29ºC	**13%**	**13%**	**45%**	**2%**

Table 1. Percentages of dorsalised phenotypes found at 18°C, 25°C and 29°C.

To further corroborate that embryos from *fs(1)N*^{12}, *fs(1)ph*^{1901} and *clos*^{1} mothers displayed dorsalised defects, we examined the expression pattern of the *zerknullt* gene (*zen*), which in wild-type stage 4 embryos is detected in a dorsal ectoderm stripe (Fig.40A) (Doyle et al., 1989). In particular, we analysed *zen* expression in embryos laid by *clos*^{1} mothers raised at 29ºC. Indeed, as shown in Table1, in this condition, we could observe that 45% embryos displayed a dorsalised phenotype beside their terminal one. Since *zen* expression is sustained at the poles by Tor activity (Helman et al., 2012), in embryos devoid of terminal activity, as the ones laid by *clos*^{1} mothers, *zen* is not expressed at the poles; this is the case for all the embryos from *clos*^{1} mutant mothers (Fig,40B). However, we observed that in some embryos *zen* expression pattern was expanded towards the ventral side (Fig,40C).

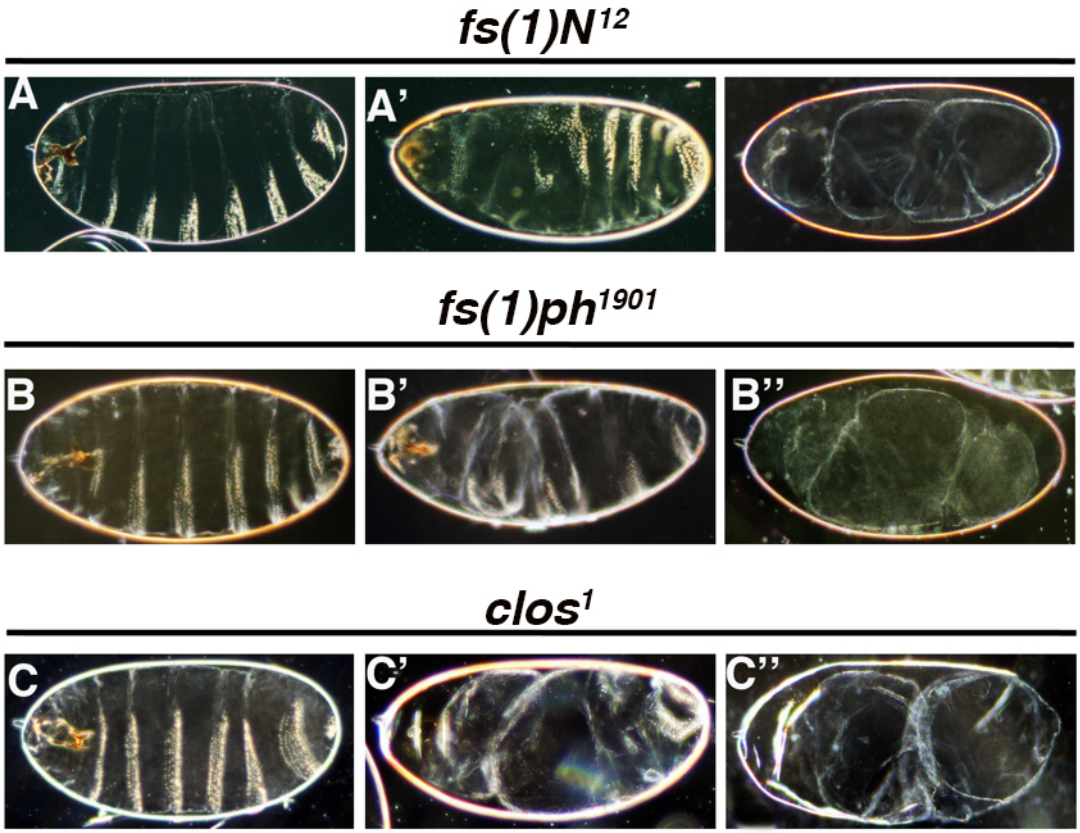

Figure.39 Phenotypes observed in *fs(1)N*^{12}, *fs(1)ph*^{1901} and *clos*^{1}. (**A-C'**) Cuticle of embryos from *fs(1)N*^{12}, *fs(1)ph*^{1901} and *clos*^{1}/*clos*^{4152} at 29°C. (**A,B,C**) Cuticle of embryos displaying a terminal phenotype (note the lack of all anterior and posterior terminal structures). (**A'B',C'**) Cuticle of a embryo displaying a terminal phenotype and a partially dorsalised phenotype (note the lack of the posterior

terminal structures and the lack of some ventral denticle belts anteriorly). (**A'',B'', C''**) Cuticle of embryoS displaying a dorsalised phenotype (note the lack of ventral denticle belts).

This result indicates an expansion of the dorsal fate in agreement with the cuticle phenotype. Having seen that *fs(1)N*^{12}, *fs(1)ph*^{1901} and *clos*^{1} flies lay embryos with a dorsalised phenotype, we wondered whether these embryos had defects in the crosslinking of their vitelline membrane. In the case of *clos*, it has been previously reported that *clos*^{1} mutant mothers lay eggs with crosslinking defects and are permeable to the neutral red dye, further indicating defects in the integrity of the vitelline membrane (Ventura et al., 2010). However, no defects in vitelline membrane cross linking or eggshell integrity have been reported in the case of *fs(1)N*^{12}, *fs(1)ph*^{1901} hemizygous females (Cernilogar et al., 2001; Ventura et al., 2010). Therefore, we extended our analysis to embryos laid by *fs(1)N*^{12}, *fs(1)ph*^{1901} and *clos*^{1} at different temperatures and we found, as for the dorsoventral phenotype, that increasing in temperature leads to an increase in neutral red permeability (Table 2). These results suggest that the increase in temperature causes defects in dorso-ventral patterning and eggshell integrity.

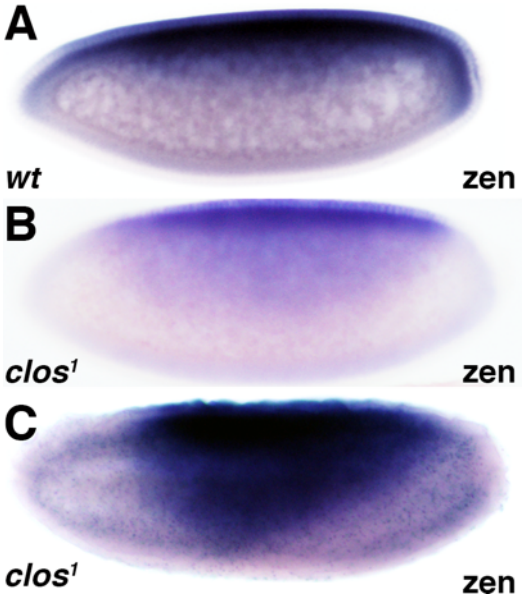

Figure.40 Expression of *zen* in embryo laid by *clos^{1}/clos^{4152}* flies raised at 29ºC (**A-C**) *In situ* hibridisation of *zerknullt* (*zen*) in a *wild type* embryo (**A**) and in embryos derived from *clos^{1}/clos^{4152}* flies raised at 29ºC (**B-C**). In these embryos, *zen* mRNA expression is not sustained at the poles reflecting lack of Tor activation (**B-C**) and, in some cases, it also expands ventrally reflecting a lack of Toll activation (**C**).

However, as previously reported (Cernilogar et al., 2001; Ventura et al., 2010), in the case of *fs(1)N^{12}* and *fs(1)ph^{1901}* dorsalised embryos and vitelline membrane permeability to neutral red were observed only in homozygosity.

	fs(1)N^{12}	*fs(1)ph^{1901}*	*clos^{1}/clos^{4152}*	*fs(1)ph^{1901}; Ph-HA/+*
18°C	**12%**	**1%**	**0%**	**/**
25°C	**18%**	**3%**	**92%**	**/**
29°C	**23%**	**15%**	**97%**	**3,5%**

Table 2. Percentages of embryos permeable to the neutral red dye.

Indeed, females carrying heteroallelic combination of *fs(1)N^{12}* and *fs(1)ph^{1901}* over their null alleles, respectively, give rise to embryos displaying only terminal defects with no impairment in eggshell integrity (Cernilogar et al., 2001; Ventura et al., 2010).To confirm that the dorsalised phenotype and the eggshell integrity defects were indeed due to a mutation in the *fs(1)ph* gene, we observed that *fs(1)ph^{1901}* females give rise to *wild type* progeny when rescued by a HA transgenic construct containing the entire Polehole sequence (Ph-HA) (Table.1 and Table.2) (Jimenez et al., 2002). Therefore, it seems that the *fs(1)N^{12}* and *fs(1)ph^{1901}* terminal mutants do not exactly behave as hypomorphs. This might suggest some dominant negative effect of these alleles although, when in hemyzigosity over a *wild type* allele, they do not give rise to any phenotypical defects in terminal or in dorsoventral patterning as expected if they were encoding for aberrant proteins behaving as dominant negatives. Thus, their genetic behaviour remains elusive although we can conclude that it is not due to a second site mutation in the chromosome.

	fs(1)N^{12}; ndΓ/+	*fs(1)ph^{1901}; ndΓ/+*	*clos^{1}/clos^{4152}; ndΓ/+*
18°C	**/**	**/**	**8%**
25°C	**/**	**/**	**41%**
29°C	**15%**	**29%**	**79%**

Table 3. Percentage of dorsalised embryos laid by *fs(1)N*^{12}, *fs(1)ph*^{1901} and *clos*^{1} in a ndl hemizygous background

Finally, we found a genetic interaction between *ndl* and *fs(1)N*^{211} ,*fs(1)ph*^{1901} and *clos*^{1} in dorsoventral patterning as the penetrance of the dorsoventral phenotype in embryos from these females increases in a Ndl hemyzigous background (Table 3).

	***fs(1)N*^{12}; ndΓ/+**	***fs(1)ph*^{1901}; ndΓ/+**	***clos*^{1}/*clos*^{4152}; ndΓ/+**
18ºC	**/**	**/**	**2%**
25ºC	**/**	**/**	**97%**
29ºC	**20%**	**24%**	**98%**

Table 4. Percentages of embryos permeable to the neutral red dye in a *ndl* hemizygous background

In the same genetic background, we also found neutral red permeability slightly increased in embryos laid by *fs(1)ph*^{1901} and *clos*^{1} females whereas this was not the case for the case of embryos laid by *fs(1)N*^{12} where permeability was slightly reduced (Table 4).

5.4 Ndl protease activity depends on Nasrat, Polehole and Closca

As already mentioned before, during mid-oogenesis, Ndl is secreted as a zymogen of 350kDa, which in late oogenesis undergoes a proteolitical processing to generate a C-terminal polypeptide of 250kDa containing the Ndl protease domain (Fig.41A) (Jimenez et al., 2002; LeMosy and Hashimoto, 2000).Since Nasrat, Polehole and Closca are involved in Ndl localisation or stabilisation, we wondered whether they might also affect Ndl processing in oogenesis. As shown in Fig.41B, a 350 kDa full length Ndl and a 250 kDa C-terminus polypeptides are detected in egg chambers from *fs(1)N* null females. Moreover, quantification of the Ndl intensity signal, compared to loading control (anti-Tubulin), suggests that Ndl levels are not dramatically reduced in *fs(1)N* null egg chambers compared to *wild type* ones. This result suggests that although Nasrat, Polehole and Closca seem to be involved in Ndl anchorage or stabilisation in the egg chamber, they are not involved in its proteolitical processing in late oogenesis.

In the transition between oogenesis and embryogenesis, generation of an active Ndl

protein requires at least two proteolytic events: the first one takes place upon egg activation and occurs independently of Nudel protease generating C-terminal 110-130 kDa polypeptides (Jimenez et al., 2002; LeMosy and Hashimoto, 2000). The second one depends on Ndl proteolitical activity itself giving rise to a 50-60 kDa doublet from the C-terminal 110-130 kDa fragment (LeMosy et al., 1998). We could detect the same proteolytic pattern although, in our hands, we observed slightly different molecular weights. It has been shown that generation of an active Ndl requires Ndl proteolitical activity. Accordingly, missense mutations in the Ndl protease domain, prevent, or at least dramatically reduce, the generation of the C-terminal 50-60kDa Ndl active form and cause the accumulation of intermediate processed polypeptides of 110-130kDa (Fig.41A) (LeMosy et al., 1998; LeMosy et al., 2000).

Therefore, we analised Ndl proteolysis in eggs laid by *fs(1)N* mutant mothers. We found that in these oocytes the C-terminal 50-60 kDa doublet is not detected similarly to oocytes laid by *ndl*^{111} females that lack Ndl proteolitical activity (Fig.42C) (LeMosy et al., 2000).

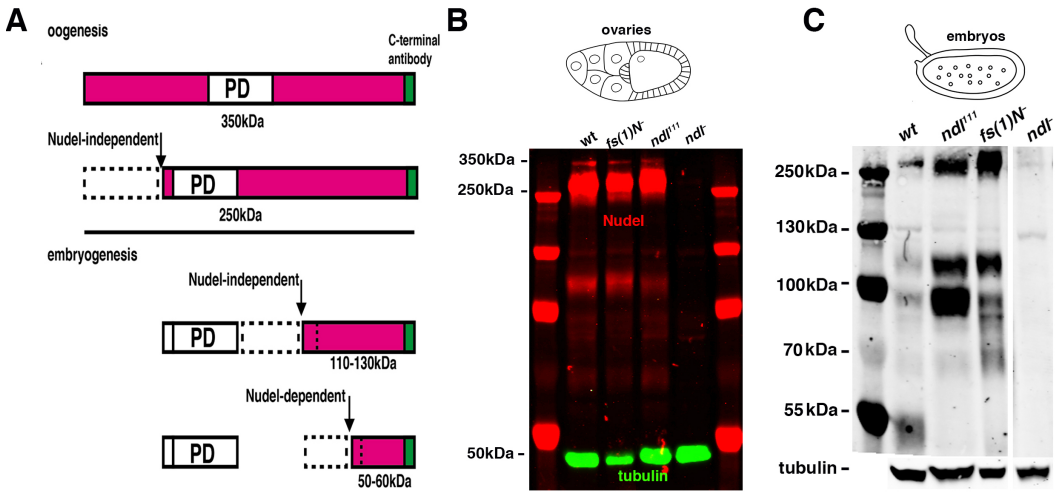

Figure.41 Ndl processing processing depends on *fs(1)N* (**A**) Schematic diagram of Nudel processing defined by Western blots adapted from LeMosy et al., 1998). (**B**) C-terminal Ndl polypeptides found in ovaries from *wt, fs(1)N*^{14}*, ndl*^{111}*/Def(3L)CH12* and *ndl*^{14}*/Def(3L)CH12* females. Ndl (in red) 350 kDa (at very low intensity) and Ndl 250kDa C-terminal polypeptide are detectable in all cases except in in ovaries derived from females *ndl*^{14}*/Def(3L)CH12*; (lane 4). *ndl*^{14} encodes for a truncated Ndl protein that is not recognised by the antibody (LeMosy et al., 2000). An antibody against Tubulin 50kDa (in green)

was used as loading control. (**C**) C-terminal Ndl polypeptides found in embryos laid by *wt, fs(1)N^{14}, ndl^{111}/Def(3L)CH12* and *ndl^{14}/Def(3L)CH12* mothers. Embryos from *fs(1)N^{14}* and *ndl^{111}* mutant females (lane 2-3), lack completely processed C-terminal polypeptides (50–60 kDa) that require functional Nudel protease. Increased levels of incomplete processed Ndl (110-130kDa) C-terminal polypeptides (the precursor of the 50-60 kDa polypetides) can be detected in embryos from *fs(1)N^{14}* and *ndl^{111}* mutant females (lane2-3) compared to *wild type* (lane1). Note that a ≈90kDa Ndl signal is detected in in eggs laid by *wt, fs(1)N^{14}, ndl^{111}/Def(3L)CH12*. An antibody against Tubulin 50kDa (bottom) was used as loading control.

Moreover, increased levels of the 110-130kDa C-terminal polypeptides can be detected in oocytes from *fs(1)N* mutant females and from *ndl^{111}* compared to *wild type* ones (LeMosy et al., 2000). Altogether, these data show that Ndl protease activity is compromised in embryos from *fs(1)N* mutant females indicating that Nasrat, Polehole and Closca have a role on Ndl function besides their role in stabilisation or localisation in oogenesis. Furthermore, Ndl proteolytic function is required for embryonic patterning but also for proper vitelline membrane crosslinking. These results suggest that the failure in Ndl protease activity detected in the embryos laid by *fs(1)N* null mutant females can account for the eggshell integrity phenotype observed in *fs(1)N*, *fs(1)ph* and *clos* null mutant females, although it may not be its only cause as these proteins might have additional roles in eggshell integrity independent of Ndl protease activity (LeMosy and Hashimoto, 2000).

5.5 Ndl activity is impaired in embryos laid by *clos^{1}* mutant mothers

The phenotypes observed in *fs(1)N^{12}*, *fs(1)ph^{1901}* and that *clos^{1}* mutant mothers revealed that Nasrat, Polehole and Closca proteins have a role in dorsoventral patterning besides their role in terminal patterning. As we have already shown that in the absence Nasrat, Polehole and Closca Ndl protease is not activated, we tested whether Ndl protease activity was also affected in embryos laid by *fs(1)N^{12}*, *fs(1)ph^{1901}* and *clos^{1}* mutant mothers. To address this issue, we analised Ndl activity in embryos laid by *clos^{1}* females at different temperatures as, at 29°C, they displayed the most highest percentage of embryos with a dorsalised phenotype and permeability to the neutral red dye (Table 1 and 2). We compared Ndl activity in these embryos and in the ones laid by *clos^{1}* females raised at 18°C which do not display defects in dorsoventral

axis specification and are not permeable to the neutral red dye suggesting that, in this condition, the Ndl protease is fully functional (Table 1 and 2).

As shown in Fig.42, we found that Ndl proteolitycal activity is not affected in embryos from *clos*^{1} mutant mothers at 18°C as Ndl dependent 50-60 kDa polypeptides are generated (compare lane1 and 2). However, in these embryos, Ndl 110-130 kDa polypeptides are more abundant than in wild type embryos suggesting that Ndl processing might be slightly affected. On the other hand, Ndl activity is strongly reduced in embryos laid by *clos*^{1} mutant mothers raised at 29°C compared to embryos from *wild type* or *clos*^{1} mutant females raised at 18°C (compare lane 3 to lanes 1 and 2). Accordingly, Ndl 110-130 kDa polypeptides are accumulated due an improper Ndl protease. However, it has to be taken into account that, although 45% of the embryos laid by these mothers are dorsalised, the remaining 55% do not display any defect in dorsoventral patterning specification indicating a functional Ndl protease activity. Therefore, in this condition, we have a heterogeneous embryonic population in which Ndl protease might be active in some of them and inactive in others.

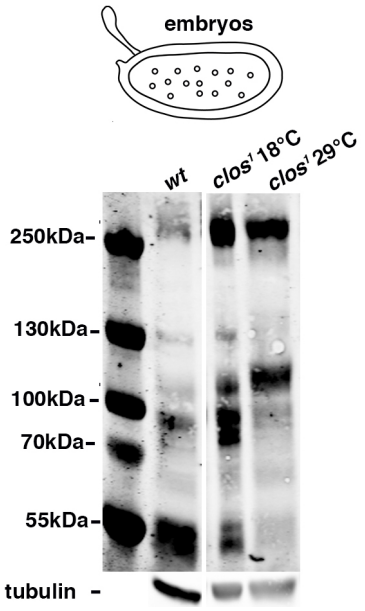

Figure.42 Ndl activity is affected in embryos from *clos*^{1} flies. C-terminal Ndl polypeptides found in embryos laid by *wild type, clos*^{1}*/clos*^{4152} raised at 18°C and *clos*^{1}*/clos*^{4152} females raised at 29°C. Completely processed C-terminal polypeptides (50–60 kD) are detectable at high levels in embryos from *wt* and *clos*^{1}*/clos*^{4152} raised at 18°C mothers (lane 1-2) but they are strongly reduced in embryos from *clos*^{1}*/clos*^{4152} females raised at 29°C (lane 3). Moreover, increased levels of incomplete processed Ndl

(110-130kDa) C-terminal polypeptides are detected in embryos laid by *clos*^{1}/*clos*^{4152} mutant mothers raised at 18°C and 29°C (lane2-3) compared *wt* (lane 1). An antibody against Tubulin 50kDa (bottom) was used as loading control.

These results show that *clos* is needed to ensure proper Ndl protease activity. Therefore, different temperatures are able to modulate different levels of Ndl activity that are responsible for the defects in eggshell integrity and dorsoventral patterning. These results show that the defects in eggshell integrity and dorsoventral patterning observed in embryos from *clos*^{1} mutant mothers are due to a lack of Ndl proteolitical activity, further corroborating the role of the Nasrat, Polehole and Closca proteins on Ndl function.

5.6 Independent domains in the Nasrat protein: charachterisation of the *fs(1)N*^{4} allele

The *fs(1)N*^{12} and *fs(1)N*^{4} point mutations were thought to specifically impair the terminal and eggshell integrity functions of Nasrat respectively as indicated by the intragenic complementation between the two alleles (Degelmann et al., 1990; LeMosy and Hashimoto, 2000) and our own observations). This result suggested that Nasrat might be a modular protein with different functional domains (Degelmann et al., 1990; LeMosy and Hashimoto, 2000). Therefore, we decided to further characterize the *fs(1)N*^{4} allele. As expected from its lack of terminal phenotype, we found that Tsl was correctly localised in egg chambers from *fs(1)N*^{4} mutant mothers (Fig.43B-B''). In the same egg chambers, we found that Ndl was also correctly accumulated at the vitelline membrane indicating that the *fs(1)N*^{4} does not affect its anchorage or stabilisation. We next examined whether the *fs(1)N*^{4} eggshell integrity phenotype might be associated with an impaired Ndl protease activity. As shown in Fig.44A, in embryos from *fs(1)N*^{4} mutant mothers we could detect the 250 kDa C-terminal Ndl polypeptide, but not the 110-130kDa fragments; instead we detected other bands that were not present in any other condition (compare lane 1 and 2).

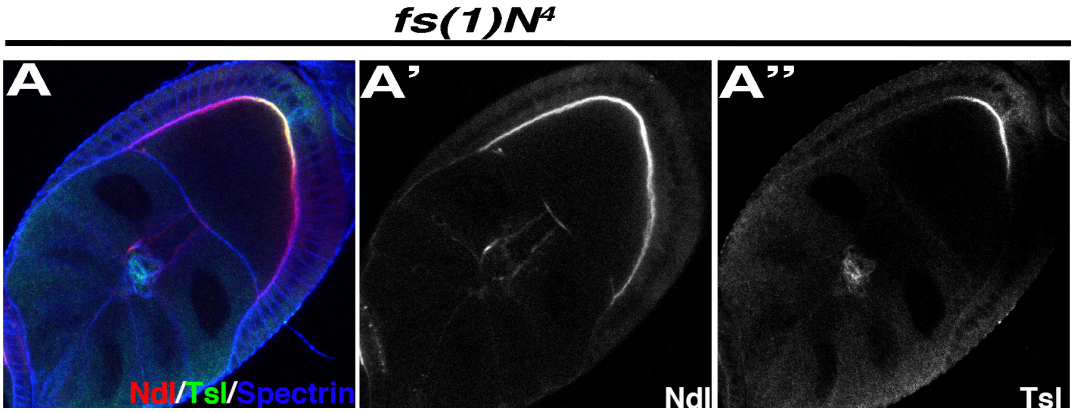

Figure43. **Ndl and Tsl localisation in *fs(1)N*^{4} egg chambers.** (**A**) The *fs(1)N*^{4} allele has an amino acid substitution (from E to V) at residue 350.(**B**) Confocal section of a *fs(1)N*^{4} stage 9 egg chamber immunostained with anti-C-Ndl (in red), anti-Tsl (in green) and anti-Spectrin (in blue) antibodies. (**B′**) Same image as (B) in the red channel showing Ndl accumulation. (**B′′**) Same image as (B) in the green channel showing Tsl accumulation.

Since cleavage of the 250 kDa polypeptide and formation of the 110-130 kDa fragments are independent of Ndl function and occur normally in the null *fs(1)N* mutants, the *fs(1)N*^{4} mutation appears to alter Ndl-independent processing that normally takes place in the absence of Nasrat function, thus preventing to assess the downstream Ndl-dependent processing of Ndl.

However, to specifically assess whether Ndl protease activity was functional in *fs(1)N*^{4} mutants we took advantage of the observation that *in vitro* egg activation is sufficient to trigger both the Ndl-dependent and Ndl-independent processing of Ndl in stage 14 *wt* oocytes (LeMosy and Hashimoto, 2000). Conversely, *in vitro* egg activation in stage 14 oocytes from *fs(1)N*^{4} mutant females was able to induce the Ndl-independent but not the Ndl-dependent cleavage of Ndl (lane 2 and 3 Fig.44B), suggesting that the Ndl activity is also impaired in the *fs(1)N*^{4} mutants.

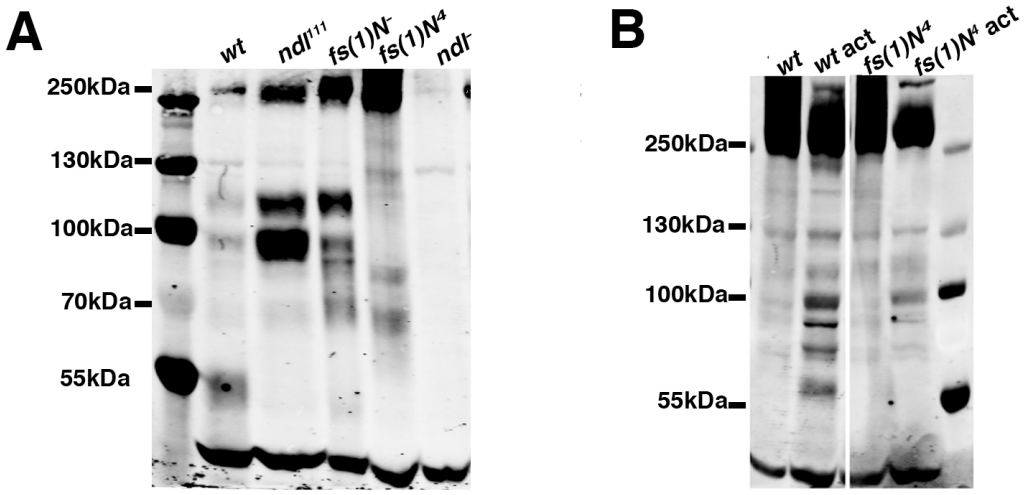

Figure.44 Ndl processing in embryo laid by *fs(1)N^4* flies. (**A**) C-terminal Ndl polypeptides found in embryos laid by *wt, fs(1)N^{14}, ndl^{111}/Def(3L)CH12, fs(1)N^{A1038}* and *ndl^{14}/Def(3L)CH12* mothers. Embryos from *fs(1)N^{14}* and *ndl^{111}* mutant females (lane 2-3), lack completely processed C-terminal polypeptides (50–60 kDa) that require functional Nudel protease and diplay increased levels of incomplete processed Ndl (110-130kDa) C-terminal polypeptides. In embryos laid by *fs(1)N^4* mutant females only the C-terminal 250 kDa Ndl polypeptide can be detected lacking C-terminal processed Ndl polypeptides of 110-130 and 50-60 kDa. Non-specific bands at the bottom were used as loading control. (**B**) C-terminal Ndl polypeptides found oocytes from *wt* and *fs(1)N^{A1038}* mutant mothers (lane 1 and 3) and from *wt* and *fs(1)N^4* activated *in vitro* for 25 minutes (lane 2 and 4). *In vitro* egg activation triggers Ndl processing in eggs from *wt* and *fs(1)N^4* mutant mothers. Completely processed 50-60 kDa C-terminal polypeptides can be detected in eggs from *wt* but not *fs(1)N^4* mutant mothers. Nevertheless in eggs from *fs(1)N^4* mutant mothers activated *in vitro* incompletely processed Ndl (110-130kDa) C-terminal polypeptides can be detected.

Finally, we mapped molecularly the *fs(1)N^4* mutation and found it to correspond to a E to V transition at residue 350 (Fig.43A). However, due to the lack of clear motifs in the Nasrat protein we can not conclude much more than assessing that the mutation lies in a separate region from the *fs(1)N^{12}* mutation.

Discussion

6.1 General discussion

The establishment of several diverse cell populations in development, requires constant coordination of cell signals among different tissues to generate well-defined structures and patterns. The arisal of such elements depend on "symmetry breaking" events which disrupt uniformity and allow the generation of cellular and tissue diversity. It has been suggested that symmetry breaking events either enhance pre-existing asymmetries or arise from inductive processes transferring asymmetries established within one group of cells to another group of cells (Roth, 1998). In any case, if the process does not take place, existing spatial information is lost and the system collapses into symmetry (Roth, 1998).

The embryonic development of the *Drosophila* constitutes a model system to study how the asymmetry is generated in oogenesis and is transmitted to the egg and future embryo. *Drosophila* oogenesis is a complex developmental process involving the coordinated differentiation of cells derived from the germ line and the somatic line. Correct execution and timing of cell fate specification and patterning events is achieved during this process by the integration of different cell-cell signaling pathways, leading to the generation of positional information inside the egg chamber and, eventually, within the oocyte that is instrumental for the establishment of embryonic polarity.

Therefore, positional information generated in the egg chamber breaks the symmetry setting up a maternal system of positional information on which further patterning is built.

6.2 Nasrat, Polehole and Closca as effectors of delayed induction

The establishment of the embryonic axis in the *Drosophila* embryo relies on four maternal systems. Anterior and posterior axes rely on morphogens (*bicoid* and *nanos*) located at the poles that will generate a gradient of activity along the embryo. In contrast, terminal and dorso-ventral system rely on cues that are generated by follicle cells in the egg chamber to induce the restricted activation of their receptors, Tor and Toll respectively.

Since follicle cells degenerate by the end of oogenesis, a mechanism must exist to maintain the positional information stable for very long periods as females can retain mature oocytes up to 15 days before fertilisation (Roth, 1998). Hence, all the information for embryonic patterning, derived from the follicular epithelium, has to be stored either in the vitelline membrane or in in the oocyte plasma membrane. Since it exists a delay between the moment when the cues are generated until they actually perform their role in embryogenesis, this mechanism has been defined as "delayed induction" (Roth, 1998). The results presented here elucidate some aspects of the molecular mechanisms that give rise to "delayed induction".

We showed that Tsl and Ndl are expressed and secreted in oogenesis and anchor to the vitelline membrane through the action of Nasrat, Polehole and Closca proteins. Therefore, terminal and dorso-ventral systems, hitherto considered independent in their extracellular pathways, have Nasrat, Polehole and Clos as common mediators. At egg activation, Tsl and, probably, Ndl translocate from the vitelline membrane to the embryonic plasma membrane to perform their yet unknown role in embryonic patterning. Moreover, at egg activation triggers the translation of maternal mRNAs as *tor* (Casanova and Struhl, 1989) and many others (Tadros and Lipshitz, 2005). In the case of the terminal system, Tsl translocation to the plasma membrane and *tor* RNA translational control under a shared trigger (egg activation) might allow the simultaneous presence of Tsl and the Tor receptor at the embryonic plasma membrane ensuring the timely activation of the Tor signalling.

Thus, retention of anchored determinants to the eggshell and their subsequent translocation to the embryonic plasma membrane might serve as a mechanism to provide spatial and temporal control of early embryonic patterning. In this scenario, Nasrat, Polehole and Closca might act as a multifunctional hub involved in the anchorage or stabilisation at the vitelline membrane of the cues of terminal and dorsoventral system and, probably, also in their translocation to the embryo plasma membrane. Thus, it might be that the mechanism of "delayed induction" relies on the Nasrat, Polehole and Closca hub at the vitelline membrane might work as a switch to orchestrate terminal and dorso-ventral signalling pathway at egg activation (Fig.45). Moreover, the here proposed new Tsl-independent function of Nasrat, Polehole and Closca in terminal signalling suggests that there are still to be identified other

components that might interact with these proteins to provide additional functions in early embryogenesis.

These findings shed further light on the vitelline membrane not just as a protective layer for the embryo but also as a highly specialised extra cellular matrix. The vitelline membrane would work as repository for asymmetric signals generated in the egg chamber ensuring the transfer of information from the egg chamber to the eggshell and, eventually, to the embryo.

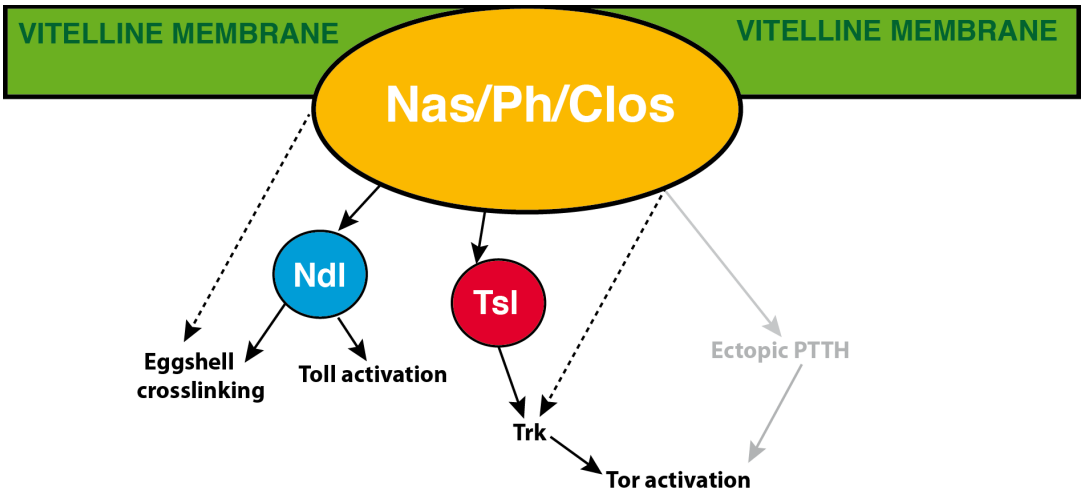

Figure.45 Nasrat, Polehole and Closca as a hub at the vitelline membrane to regulate different functions

6.3 Nasrat, Polehole and Closca in vitelline membrane cross linking and embryonic patterning

fs(1)N, fs(1)Ph and *clos* genes encode for proteins related among them by sequence but lacking any known domain. As these proteins are mutually required for their proper localisation at the vitelline membrane, it is very likely that they interact among them forming a complex. Unfortunately, we (data not shown) and others (Jimenez et al., 2002) could not detect any physical interaction among them.

While the *fs(1)N, fs(1)Ph* and *clos* were known to be involved, in a still unknown way, in terminal signalling and in the crosslinking of the vitelline membrane, now we have

indication that shed light on their function suggesting that they have multiple roles. In the case of Tsl, Nasrat, Polehole and Closca proteins are known to play a crucial a role in its anchorage or stabilisation, as Tsl cannot be detected in egg chambers from the *fs(1)N, fs(1)Ph* and *clos* mutant females (Jimenez et al., 2002). Here, we showed that Nasrat, Polehole and Closca are involved, at least partially, also in the anchorage or stabilisation of Ndl at the nascent vitelline membrane. Although Ndl is only partially mislocalised in the absence of Nasrat, Polehole and Closca, these proteins have a specific role in its anchorage or stabilisation at the vitelline membrane. Furthermore, we showed that they also impinge on Ndl localisation in late oogenesis between follicle cells together with other vitelline membrane proteins. This observation might indicate that Nasrat, Polehole and Closca have a role in late oogenesis that remains to be elucidated.

The process and the enzyme(s) (probably a peroxidase) (Margaritis, 1985; (Petri et al., 1976)). involved in vitelline membrane crosslinking are not known (LeMosy and Hashimoto, 2000) but Nasrat, Polehole, Closca and Ndl are clearly involved in this process (Cernilogar et al., 2001; Jimenez et al., 2002; LeMosy and Hashimoto, 2000). We showed that Nasrat, Polehole and Closca are required for Ndl proteolitical activity besides their role in its localisation or stabilisation. Since, as already mentioned above, Ndl provides the proteolitical function necessary for proper embryonic dorsoventral polarity and vitelline membrane integrity, these results suggest that the role of Nasrat, Polehole and Closca in dorso-ventral patterning and vitelline membrane integrity is mediated by Ndl protease activity. Indeed, we showed that in embryos laid by $clos^{1}$ mutant females Ndl protease activity is temperature sensitive indicating that defects in dorso-ventral patterning and vitelline membrane integrity observed at higher temperature are due to a lack of Ndl protease activity. Likewise, embryos from $fs(1)N^{12}$ and $fs(1)Ph^{1901}$ mutant females also display defects in dorso-ventral patterning and vitelline membrane integrity suggesting an improper Ndl proteolytic function although to a lesser extent compared to the ones laid by $clos^{1}$ mothers.

As Ndl protease activity is required for vitelline membrane cross linking, it is tempting to speculate that Ndl might mediate the role of Nasrat, Polehole and Closca in vitelline membrane crosslinking as we showed that in embryos from *fs(1)N, fs(1)Ph* and *clos* females, Ndl protease activity is compromised. However, while lack of Ndl protease

function in Class II *ndl* alleles, cause defects in vitelline membrane integrity and dorso-ventral patterning, embryos laid by *fs(1)N, fs(1)Ph* and *clos* mutant females collapse upon treatment with bleach or are arrested very early in embryogenesis. Similarly, embryos laid by females carrying a *ndl* Class I mutant alleles give rise to embryos that collapse or arrest in early embryogenesis due to an additional role of the non-protease domain of the Ndl protein in the integrity of the vitelline membrane (LeMosy and Hashimoto, 2000). Therefore, lack of Ndl proteolitical activity in embryos lacking Nasrat, Polehole and Closca does not explain the "collapse" phenotype observed in these embryos. This result suggests that Nasrat, Polehole and Closca have an additional role on vitelline membrane integrity besides their role in regulating Ndl protease activity. Being these proteins vitelline membrane components, this additional role might consist in a structural role in the vitelline membrane. Indeed, although vitelline membrane components as sV23 and VM32E localise correctly in egg chambers from *fs(1)N* mutant females in confocal microscopy, no studies have been carried out at the ultrastructural levels in these egg chambers which might indicate structural defects that cannot be detected by optical microscopy. Alternatively, the additional role of Nasrat, Polehole and Closca might be mediated by the non-protease Ndl region. Indeed, as mentioned in the introduction, Ndl non-protease region might be also required for the cross linking or might have a structural role for the vitelline membrane (LeMosy and Hashimoto, 2000). Therefore, these proteins might have two roles in regulating Ndl: a first one involved in regulating the function of Ndl non-protease region and a second one on its protease activity. Thus, in embryos laid by *fs(1)N, fs(1)Ph* and *clos* females, both functions would be affected giving rise to embryos that collapse. On the other hand, the terminal alleles $fs(1)N^{12}$, $fs(1)Ph^{1901}$ and $clos^{1}$ would affect only the proteolytic Ndl function giving rise to embryos with defects in dorso-ventral patterning and vitelline membrane integrity that do not collapse and sustain embryonic development.

To distinguish between these two scenarios, further studies on vitelline membrane integrity and cross linking are needed. In particular, it would be crucial to distinguish whether the "collapse" and the "vitelline membrane integrity" phenotypes (permeability to neutral red) are affecting two independent processes. Indeed, it might be the case that the permeability to neutral red reveals subtle defects in vitelline

membrane cross linking while the collapse might be due to a complete absence of cross linking among vitelline membrane proteins. Moreover, it would be important to charachterise whether eggs permeable to neutral red are capable to progress embryonic development, indicating whether even subtle impairments in the eggshell compromise embryonic development. This information might also shed light on the role of Ndl in embryonic patterning and vitelline membrane integrity. Indeed, despite of many analyses on Ndl activity, it still remains an open question whether its function in dorso-ventral patterning and vitelline membrane integrity are completely independent of each other or not (Stein and Stevens, 2014). Indeed, on the one hand, as proper Spätzle activation requires *pipe* dependent sulfatated component of the vitelline membrane (Zhang et al., 2009b), Ndl protease activity might ensure the proper localisation or activation of these components, thus having an indirect role on dorso-ventral axis specification (LeMosy and Hashimoto, 2000). On the other hand, Ndl might have two distinct function localised in different embryonic domains: vitelline-membrane-bound Ndl might be responsible for the cross linking of the vitelline membrane while, later, plasma-membrane bound Ndl protease might be responsible for embryonic patterning.

6.4 Different functional domains in Nasrat, Polehole and Closca

Although the Nasrat, Polehole and Closca proteins lack of any domain that could suggest their function, genetic data suggested that they might have different functional domains. This idea was also based on the identification of specific point mutations (namely *fs(1)N*^{12} and *fs(1)Ph*^{1901}) that do not abolish vitelline membrane integrity but impinge on terminal signalling (Degelmann et al., 1990). Afterwards, the idea of independent domain was weakened by the discovery of the *clos* gene. Indeed, females bearing a point mutation in *clos* (*clos*^{1}) lay embryos that display defects in terminal patterning but also in vitelline membrane cross-linking suggesting the lack of clear independent domains (Ventura et al., 2010). The data presented here further reinforce this notion. Indeed, embryos from *fs(1)N*^{12} and *fs(1)Ph*^{1901} homozygous mutant mothers display defects in vitelline membrane integrity (and dorso-ventral patterning) besides their terminal phenotype. These observations suggest the lack of distinct domains, one required for embryonic patterning and the other for vitelline membrane crosslinking.

The *fs(1)N*^{12}, *fs(1)Ph*^{1901} and *clos*^{1} might be null mutants respect to their terminal function but hypomorph mutants affecting the role of Nasrat, Polehole and Closca in the integrity of the vitelline membrane and dorso-ventral patterning. Thus, the effect of the *fs(1)N*^{12}, *fs(1)Ph*^{1901} and *clos*^{1} mutant in dorso-ventral and terminal patterning might be due to an improper Ndl proteolitical activity and to their new Tsl independent role in terminal signalling. However, trans-allelic combination of *fs(1)N*^{12} and *fs(1)ph*^{1901} over their null alleles, respectively, gives rise to embryos showing only the terminal phenotype, but not the dorsalised phenotype and vitelline membrane integrity defects (Cernilogar et al., 2001; Ventura et al., 2010). This result indicates that *fs(1)N*^{12} and *fs(1)ph*^{1901} do not behave exactly as hypomorph mutants respect to their function in vitelline membrane cross linking and dorso-ventral patterning and their behaviour still remain elusive.

On the other hand, the data obtained using the *fs(1)N*^{4} allele suggest the presence of different domains in the Nasrat protein as confirmed by the intragenic complementation of *fs(1)N*^{4} allele over the *fs(1)N*^{12} terminal allele (Degelmann et al., 1990). Furthermore, we have shown that in *fs(1)N*^{4} mutant egg chambers Tsl is correctly localised or stabilised at the vitelline membrane suggesting that this mutation affects a specific Nasrat domain which is not required for its anchorage or stabilisation. Moreover, intragenic complementation between *fs(1)N*^{4} and *fs(1)N*^{12}, shows that the *fs(1)N*^{4} allele does not affect either the new Tsl-independent function of Nasrat, Polehole and Closca.

We have also shown that Ndl accumulates correctly in *fs(1)N*^{4} egg chambers indicating that this mutation does not affect Ndl anchorage or stabilisation at the vitelline membrane. Nevertheless, in embryos from *fs(1)N*^{4} mothers Ndl does not undergo proteolytic cleavage remaining as a 250kDa C terminal polypeptide as generated in oogenesis. These results might suggest that the *fs(1)N*^{4} affects a domain required for the structural role in vitelline membrane integrity of Nasrat, Polehole and Closca. This result is also difficult to reconcile with the one obtained in embryos from *fs(1)N* null females where, while Ndl independent cleavage was taking place, we found an improper Ndl dependent proteolytic activation. Nevertheless, *in vitro* activation of mature oocytes from *fs(1)N*^{4} mutant mothers triggered Ndl independent processing, giving rise to a 110-130 kDa C-terminal polypeptide but was not sufficient to induce

proper Ndl protease activity. These observations suggested that *fs(1)N*^{4} might, somehow, be required for a proper egg activation and, hence, Ndl independent cleavage. However, as for the case of the *fs(1)N*^{12} allele, the genetic behaviour of the *fs(1)N*^{4} allele remains elusive as it affects a process, *i.e.* Ndl independent processing, that is not affected in *fs(1)N* null mutants. However, while in *fs(1)N* null mutants Polehole and Closca do not localise correctly at the vitelline membrane, it is not known whether the *fs(1)N*^{4} mutation affects their localisation. An intriguing possibility is that the the *fs(1)N*^{4} encodes for an aberrant Nasrat protein that by the interaction with Polehole and Clos, affects the structural stability of the vitelline membrane preventing egg activation in the passage through the oviduct. Therefore, in eggs laid by *fs(1)N*^{4} mothers the induction of egg activation *in vitro* might be able to bypass the structural defects in the vitelline membrane by forcing the Ca^{2+} influx.

6.5 Tsl translocation from the vitelline membrane to the embryo plasma membrane

As already mentioned in the introduction Tsl has a MACPF domain, a domain found in proteins always associated with the plasma membrane. Hence, Tsl localisation at the inner side of the vitelline membrane was difficult to reconcile with its molecular features (Stevens et al., 2003). In this context, the finding that Tsl also accumulates at the embryonic plasma membrane fits well with its MACPF domain.

Our results suggest that, at the beginning of embryogenesis, probably at of egg activation, Tsl translocates from the eggshell to perform its still elusive role at the embryo plasma membrane. (Fig.46A-D) Unfortunately, we could not dissect Tsl translocation at the moment of egg activation due to technical difficulties. Indeed, the induction of egg activation *in vitro* sticks together the vitelline membrane to the oocyte membrane preventing its removal and, then, Tsl detection. To overcome this caveat, a fluorescently tagged Tsl protein might allow to visualise the process *in vivo* avoiding vitelline membrane removal. Although different members of the lab generated several GFP-tagged Tsl construct unfortunately, in all the cases, Tsl function was lost upon fusion with GFP (A.Casali, 2000 Ph.D Thesis and Nicolás Martìn personal communication). However, different studies suggest that, at egg activation, vitelline

membrane and embryonic plasma membrane are tightly attached and this might prevent to visualise Tsl translocation *in vivo* from one membrane to another (LeMosy and Hashimoto, 2000).

However, we can conclude that that Tsl translocation does not depend on egg activation events downstream of *sarah*. The trigger that initiates egg activation and vitelline membrane crosslinking is thought to be an entry of Ca^{2+} from the oviduct. Moreover, the initial binding of some MACPF proteins as Perforins to the cell surface is Ca^{2+} dependent (Voskoboinik et al., 2005). Therefore, we can envisage two scenarios. In the first one, the entry of calcium at egg activation might cause conformational changes in the Tsl protein allowing its release from the vitelline membrane in the perivitelline space and, thus, to the embryonic plasma membrane. Alternatively, changes in the vitelline membrane, due to its crosslinking, might affect the interaction between vitelline membrane proteins (as Nasrat, Polehole and Closca) and allow the release of Tsl from the vitelline membrane and its interaction with the plasma membrane.

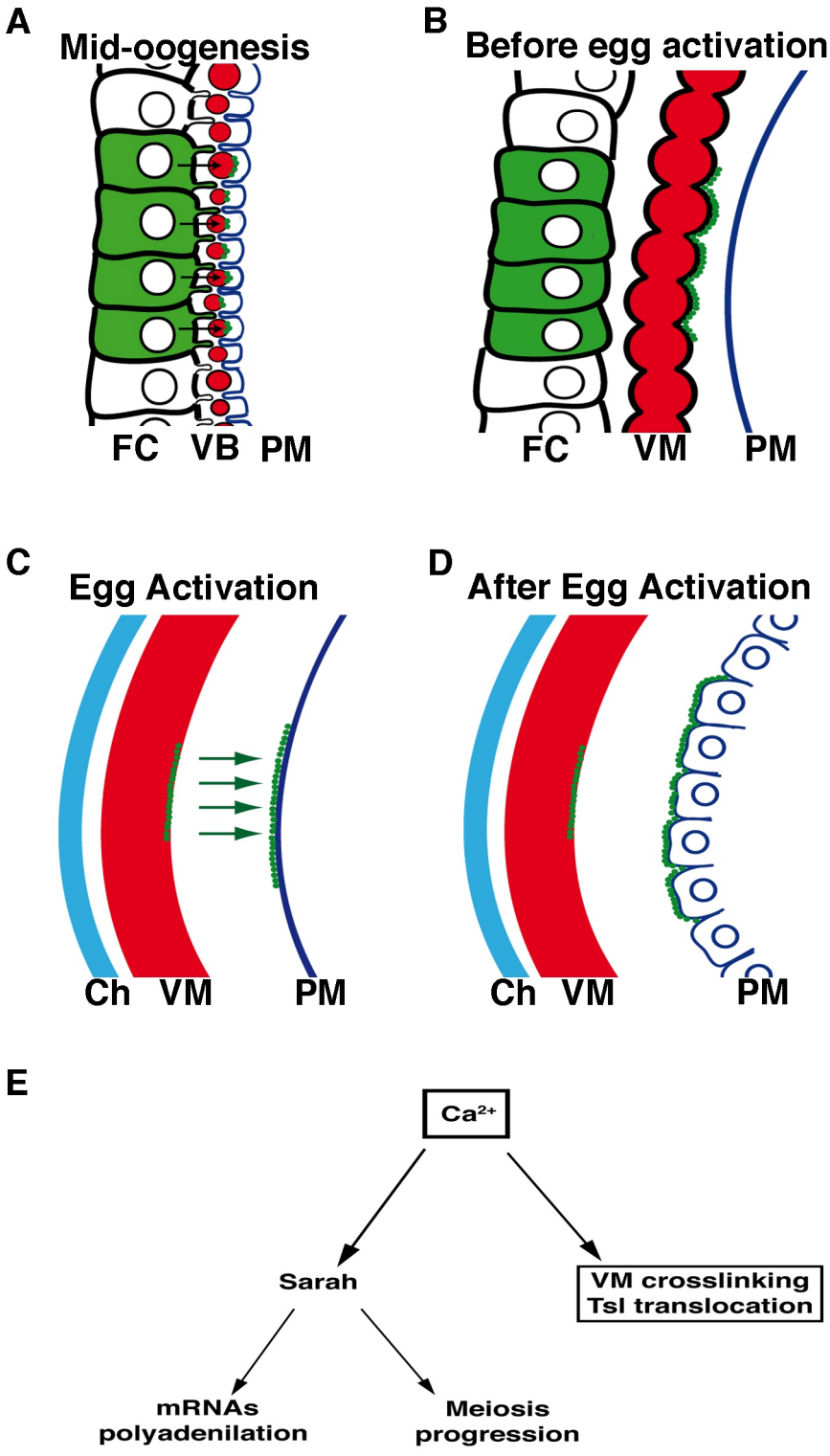

Figure.46 Schematic diagram of Tsl translocation (A) Around stage 9 of oogenesis, Tsl (in green) is secreted (arrows) from a subpopulation of follicle cells (FC) located at both ends of the oocyte and incorporated into the vitelline bodies (VB, in red), thus ensuring that the spatial asymmetry is transmitted from the ovary to the eggshell (Tsl is depicted in the inner surface of the vitelline membrane, as indicated by (Stevens et al., 2003), probably anchored through an interaction with the

Nasrat/Polehole/Closca complex. (**B**) Around stage 10-11 of oogenesis, Vitelline Bodies fuse forming a continuous Vitelline membrane (VM in red) surrounding the oocyte. Tsl (in green) remains anchored in the inner surface of the vitelline membrane until egg activation occurs. (**C**) Upon egg activation, Tsl (in green) translocates (arrows) from the vitelline membrane (VM in red) to the oocyte plasmatic membrane (PM in blue). (**D**) After egg activation, Tsl (in green) is found both at the vitelline membrane (VM in red) and at the embryonic plasma membrane (PM in blue), where it acts as the local determinant for the activation of the Tor receptor during early embryogenesis. (**E**) Egg activation takes place as mature oocytes pass through the oviduct; there, a calcium influx triggers many physiological changes such polyadenylation of maternal mRNAs, progression of meiosis and cross-linking of the vitelline membrane according to (Sartain and Wolfner, 2013). As for the cross-linking of the vitelline membrane, Tsl accumulation at the plasma membrane is *sra*-independent suggesting that either the calcium influx at egg activation or the vitelline membrane crosslinking could trigger Tsl translocation from the eggshell.

Since, it is not possible to distinguish between these two possibilities, we suggest that the likely trigger for Tsl translocation is either the calcium influx at egg activation or the vitelline membrane crosslinking (Fig.46E). Future studies on the Tsl structure will elucidate its attachment to the plasma membrane as, unfortunately, sequence homology between Tsl and other MACPF proteins is very low preventing any prediction of the Tsl structure.

However, Tsl is very likely to play its role at the embryo plasma membrane. Indeed, the *tsl-HA* transgene, even though encodes for a Tsl protein that is functional in regulating developmental timing, is not functional in the context of the embryonic terminal patterning. We have found that the Tsl-HA protein localise correctly at the vitelline membrane but it is not able to translocate to the embryo plasma membrane. Therefore, lack of functionality of the Tsl-HA protein is likely to be due to its inability to translocate to the embryonic plasma membrane. These results strongly suggest that the Tsl protein plays its role at the embryonic plasma membrane rather than the vitelline membrane as previously suggested and further strenghten Tsl translocation as an important step in Tor activation.

It has been suggested that the role of Tsl might act as a "membrane-bound protein necessary to nucleate an as yet unidentified protease complex" (see introduction) (Furriols and Casanova, 2003). Accordingly, restricted accumulation of Tsl at the poles of the embryonic plasma membrane poles might provide an asymmetrical signal to recruit a uniformly localised protease to locally activate Trk. Alternatively, it has been proposed that Tsl bound to the vitelline membrane facilitates the secretion of Trk from the embryo (see introduction) (Duncan et al., 2014). In this scenario, the accumulation of the Tsl protein at the embryonic plasma membrane corroborates this hypothesis and

strengthen the possibility that plasma membrane-bound Tsl might act as a MACPF protein allowing Trk secretion via the formation of a pore or a membrane damaging mechanism (johnosn2015). However, the data available at the moment are still too scarce to ascertain the precise mechanism of action of Tsl.

6.6 A new Tsl-independent role of Nasrat, Polehole and Clos in terminal signalling

Nasrat, Polehole and Closca are clearly involved in vitelline membrane integrity as females carrying null mutations in these genes produce flaccid eggs that collapse upon treatment with bleach. These proteins are also clearly involved in anchoring Tsl at the vitelline membrane although the role of *fs(1)N*^{12}, *fs(1)Ph*^{1901} and *clos*^{1} in terminal patterning remains poorly understood. For many years the emphasis has been put on the role of these proteins on Tsl indicating that Nasrat and Polehole proteins have an additional role in the terminal system besides the anchorage or stabilisation of Tsl and it was proposed that they were required for "Tsl to exert its function" (LeMosy, 2003; Stevens et al., 2003)

Here, we have found that the additional terminal function of Nasrat, Polehole and Closca in the terminal patterning does not consist in promoting the translocation of Tsl from the vitelline membrane to the embryonic plasma membrane. We have also tested whether their additional function might be Tsl-independent taking advantage of the fact that ectopic expression in the embryo of PTTH is able to activate Tor even in the absence of Tsl. The observation that PTTH is not able to activate Tor in embryos laid by *fs(1)Ph*^{1901} mutant females suggests that Nasrat, Polehole and Closca have an additional function on the terminal system that is independent of Tsl. Since it has been shown that two independent carboxy-terminal fragments of Trk retain the activity to activate Tor independently of *tsl*, *fs(1)N*^{12} and *fs(1)Ph*^{1901} (casali2001), thus, the additional function of Nasrat, Polehole and Closca on terminal signalling is likely to be acting on Trk and PTTH.

So what could be the common process in Trk and PTTH that is regulated by Nasrat, Polehole and Closca? As described above, Trk and PTTH belong to a group of ligand that have a cysteine knot motif (McDonald and Hendrickson, 1993; Murray-Rust et al.,

1993). This class of ligands are produced as pro-ligands that are cleaved giving rise to the active form. Accordingly, in S2 cells, it has been shown that Trk cleavage is mediated by Furin1 and Furin2 (Henstridge et al., 2014; Johnson et al., 2015). On the other hand, PTTH has many Furin putative cleavage sites along its sequence although it has not been shown whether PTTH is actually processed *in vivo* (Smith and Rybczynski, 2011).

Therefore, we envisage the possibility that Nasrat, Polehole and Closca are required for the cleavage of Trk and PTTH that would be necessary for the activation of both ligands. In this scenario an intriguing possibility is that Trk and PTTH activation is controlled by the Furin1 and Furin2 proteases through the activity of Nasrat, Polehole and Closca. Therefore, these proteins might activate proteolitical activity of Furins driving the activation of Trk and PTTH. Removal of the pro-domain would generate the active form of PTTH which is already functional and able to bind Torso. On the other hand, Trk would still require another step of activation mediated by Tsl ensuring restricted activation of Tor at the poles.

In contrast to this hypothesis, it has to be mentioned that Furins are thought to act intracellularly while Nasrat, Polehole and Closca are component of the vitelline membrane so they are unlikely to have a role in activating intracellular Furins. However, in vertebrates, Furins are thought to be associated with the oocyte plasma membrane driving the cleavage of proteins that are incorporated in the vitelline envelope that covers the oocyte (Litscher and Wassarman, 2007; Wassarman et al., 2001). Accordingly, in *Drosophila*, it has been shown that vitelline membrane proteins as sV23 and sV17, have Furin consensus cleavage sites (Pascucci et al., 1996; Waring, 2000). Moreover, flies where Furin1 has been downregulated in all follicle cells lay flaccid eggs that collapse upon bleach treatment due to defects in vitelline membrane non-disulfide cross linking (Marc Furriols unpublished observation). These observations suggest that the involvement of Furins in the processing and assembly of eggshell components might be conserved in evolution. In this scenario, Furin1 and Furin2 might be associated with the oocyte plasma membrane or the perivitelline space and, upon the activity of Nasrat, Polehole and Closca, might process Trk and ectopic PTTH (Fig.47).

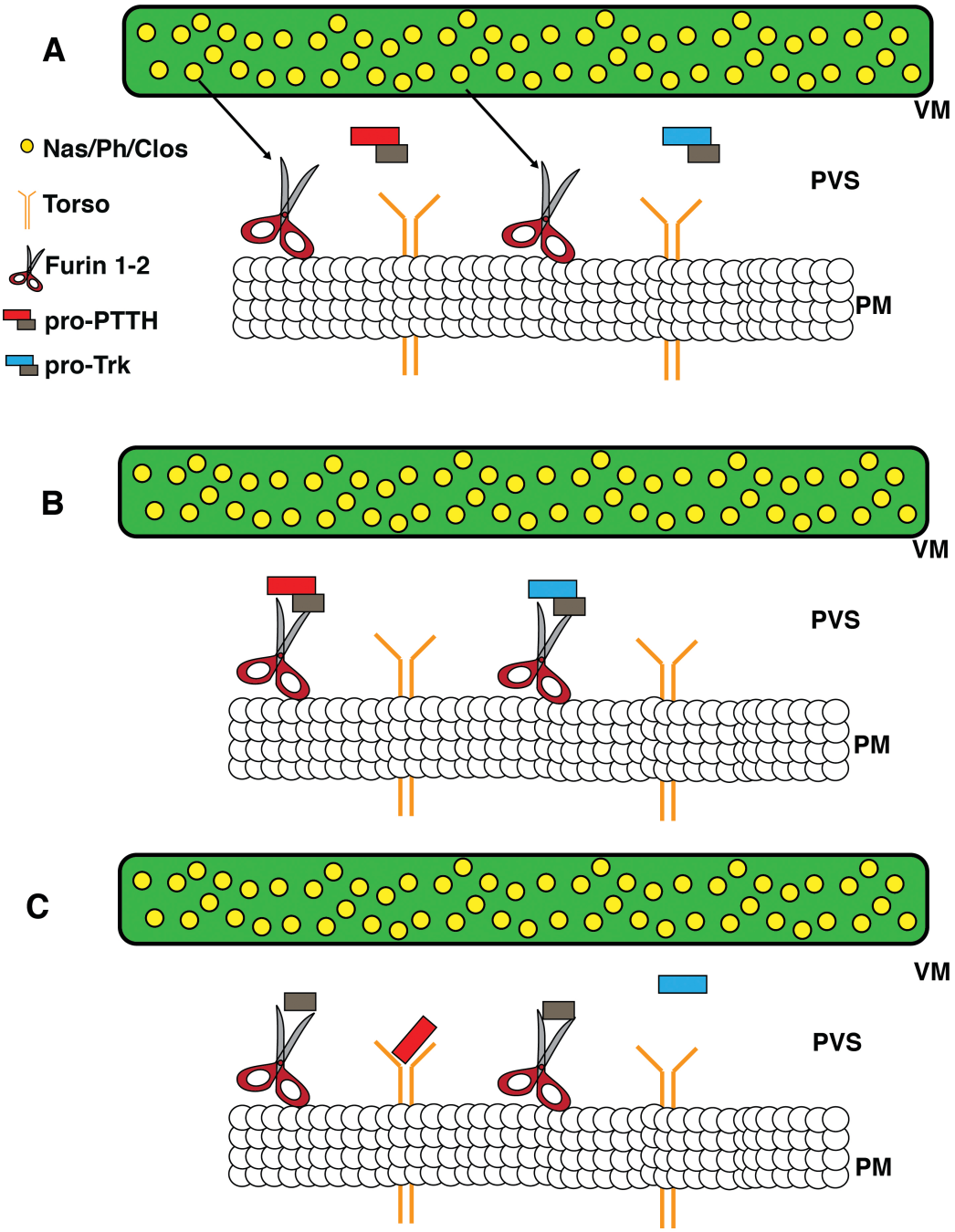

Figure.47 A new Tsl-independent function for Nasrat, Polehole and Closca in terminal signaling. Schematic diagram of the interaction between Nasrat, Polehole and Closca at the vitelline membrane (in yellow) and furin (as scissors) in the processing of the pro-domain (in grey) of PTTH (in red) and Trk (in purple), VM (Vitelline Membrane), PVS (Perivitelline Space), PM(Plasma membrane). **A**) Nasrat, Polehole and Closca activate Furins located extracellularly at the embryo plasma membrane or at the perivitelline space. **B**) Activated Furins remove the prodomain (in grey) in Trk and in PTTH. **C**)

Removal of the prodomain generates the active form of PTTH (in red) which is already functional and able to bind Torso (in orange) while Trk still needs another step of activation probably mediated by Tsl.

Conclusions

7. Conclusions

1. The Tsl protein druing oogenesis accumulates at the forming vitelline membrane and translocates to the embryo plasma membrane in early embryogenesis, probably upon egg activation.

2. Tsl accumulation at the plasma membrane does not depend on *tor* or *trk.*

3. Tsl accumulation at the plasma membrane has a functional relevance for terminal patterning.

4. Nasrat, Polehole and Closca are involved in dorso-ventral axis specification.

5. The role of Nasrat, Polehole and Closca proteins in dorso-ventral patterning and vitelline membrane integrity is mediated, at least in part, by Ndl protease activity.

6. During oogenesis, Nasrat, Polehole and Closca, are required for Ndl anchorage or stability at the vitelline membrane.

7. Nasrat, Polehole and Closca have a Tsl-independent role in terminal signalling.

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Appendix I

A common step transfers dorsoventral and terminal information from ovary to embryo in *Drosophila*

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Abstract

The *Drosophila* eggshell is an extracellular matrix that confers protection to the egg and also plays a role in transferring positional information from the ovary to pattern the embryo. Among the constitutents of the *Drosophila* eggshell, Nasrat, Polehole and Closca form a group of proteins related by sequence, secreted by the oocyte and mutually required for their incorporation into the eggshell. Besides their role in eggshell integrity, Nasrat, Polehole and Closca are also required for embryonic terminal patterning by anchoring or stabilising Torso-like at the eggshell. Here we show that they are also required for dorsoventral patterning, thereby unveiling that the dorsoventral and terminal systems, hitherto considered independent, share a common extracellular mechanism. Furthermore, we show that Nasrat, Polehole and Closca are required for proper Nudel activity, a protease acting both in embryonic dorsoventral patterning and eggshell integrity, thus providing a mechanism for the role of Nasrat, Polehole and Closca. We propose that a Nasrat/Polehole/Closca complex acts as a multifunctional hub to anchor various proteins synthesized at oogenesis, ensuring their spatial and temporal restricted function.

Key words: *Drosophila*/Nasrat/Closca/Polehole/Nudel/ Vitelline membrane/dorsoventral patterning.

Introduction

Extracellular matrices (ECMs) exert a dual role in cell biology. On the one hand, they confer a wide range of specific environments that provide distinct kinds of physical support or protection. On the other hand, they also play a key role in cell signalling as they participate in growth factor storage or modification (Rozario and DeSimone, 2010). A particular scenario in which to address the diverse roles of ECMs is that of *Drosophila* oogenesis, a process in which a highly specific ECM, namely the eggshell, is built in parallel with the development of the oocyte

The *Drosophila* eggshell consists of five morphologically distinct layers. The innermost layer closest to the oocyte is the vitelline membrane, which confers rigidity and protection to the egg but also plays a role in embryonic patterning. During oogenesis, the somatic follicle cells surrounding the oocyte secrete the four major structural components of the vitelline membrane (sV17, sV23, Vm32E, and Vm34C) (Waring, 2000) (Pascucci et al., 1996) (Andrenacci et al., 2001) (Cernilogar et al., 2001). The assembly of these proteins in the vitelline membrane requires their stage-specific proteolytic processing and cross-linking among them to render the membrane insoluble. The vitelline membrane also receives contributions from the oocyte. *fs(1)Nasrat* (*fs(1)N*), *fs(1)polehole* (*fs(1)ph*), and *closca* (*clos*) encode a group of extracellular germline proteins that are secreted from the oocyte and are mutually required for their incorporation into the vitelline membrane (Jimenez et al., 2002) (Ventura et al., 2010). Females mutant for null alleles of *fs(1)N*, *fs(1)ph* or *clos* genes produce fragile eggs that collapse after laying due to defects in vitelline membrane cross-linking (Cernilogar et al., 2001; Ventura et al., 2010).

At the same time, the ovary provides the oocyte with the basic patterning information that will specify the body plan of the future embryo along the anteroposterior and dorsoventral axes. The anterior and posterior determinants, bicoid and nanos respectively, are deposited within the oocyte, where they accumulate asymmetrically. In contrast, terminal and dorsoventral systems rely on signals secreted by the embryo that activate the Torso (Tor) and Toll (Tl) receptors in restricted domains of the embryonic membrane (for a review on *Drosophila* early embryonic patterning see (St Johnston and Nusslein-Volhard, 1992). The terminal and dorsoventral systems appear to be tightly coupled to the vitelline membrane (Stevens et al., 2003; Zhang et al., 2009a); (Jimenez et al., 2002); (Ventura et al., 2010). Thus, in the terminal system, activation of the Tor receptor at the embryonic poles by its ligand Trunk (Trk) requires fully functional components of the vitelline membrane. In this regard, there are specific mutants for each of the *fs(1)N*, *fs(1)ph* and *clos* genes that ensure eggshell formation, thus allowing embryogenesis to proceed, but impair the activation of the Tor receptor and thus embryos do not develop their terminal regions (Degelmann et al., 1990).

Likewise, in the dorsoventral system, the Nudel (Ndl) protease is required both for the activation of the Tl receptor in the ventral part of the embryo and for vitelline membrane integrity (LeMosy and Hashimoto, 2000). Indeed, *ndl* mutant alleles fall into two phenotypic classes. Eggs laid by females carrying Class I alleles (associated with the absence or aberrations of the protein (LeMosy et al., 2000) collapse or arrest in early embryogenesis. In contrast, embryos from females carrying Class II alleles (consisting of missense mutations in the protease domain (Hong and Hashimoto, 1996)) complete embryogenesis but have defects in vitelline membrane cross-linking and are dorsalised. Therefore, Ndl exerts a proteolytic function involved in embryonic dorsoventral axis specification and vitelline membrane crosslinking.

In the case of terminal signalling, while the complete process that leads to Tor receptor activation remains unclear, we are beginning to unravel how the process is linked to the vitelline membrane. In particular, the work of many laboratories has shown the following: 1) Torso-like (Tsl), a protein secreted by the follicle cells (Martin et al., 1994; Savant-Bhonsale and Montell, 1993), is required for Trk-mediated activation of the Tor receptor (Casali and Casanova, 2001; Furriols and Casanova, 2003); (Johnson et al., 2015); 2) Tsl first accumulates at the vitelline membrane during oogenesis and later translocates to the embryonic plasma membrane, probably at egg activation (Mineo et al., 2015); and 3) Nasrat, Polehole and Clos proteins are required either for the anchoring or stabilization of Tsl at the vitelline membrane (Jimenez et al., 2002); (Ventura et al., 2010) (Stevens et al., 2003)

Here we further examined the role of Nasrat, Polehole and Clos proteins in vitelline membrane integrity and cell signalling. We found that embryos from *clos*^{1} females also show defects in dorsoventral patterning, thereby unveiling that the dorsoventral and terminal systems, hitherto considered independent, share a common mechanism in their extracellular pathways. Furthermore, we show that Nasrat, Polehole and Clos are required for proper Ndl localisation and function, thus providing a mechanism for the functions of these three proteins in vitelline membrane integrity and dorsoventral patterning. We propose that Nasrat, Polehole and Clos constitute a multifunctional hub for the proper anchorage and activity of extracellular signalling molecules, ensuring

their spatial and temporal restricted function in order to ensure coordinated embryonic axis specification.

Results

clos mutants show defects in dorsoventral patterning

fs(1)N, *fs(1)ph* and *clos* null mutations cause eggshell integrity defects that lead to egg collapse and failure to sustain embryonic development. It is therefore not possible to assess the requirement of these genes in embryonic patterning from the progeny of these null mutant females. Besides, only defects in the terminal system patterning have been reported in the progeny of mutant females for the $fs(1)N^{211}$, $fs(1)ph^{1901}$ and $clos^{1}$ alleles that allow for embryonic development (Degelmann et al., 1990; Jimenez et al., 2002; Ventura et al., 2010). However, we extended the previous analyses on the embryos laid by $clos^{1}$ mutant females at various temperatures. When raised at 18ºC, $clos^{1}$ females lay embryos that display a terminal phenotype (Ventura et al., 2010). However, when raised at 29ºC, 45% of embryos had a dorsalised phenotype displaying a loss of ventral structures at the expense of dorsal ones (Fig. 1 C-D), in addition to their terminal defects (Table1). We confirmed the defects in dorsoventral patterning by examining the expression pattern of *zerknullt* (*zen*), which is detected in a dorsal ectoderm stripe in wild-type stage 4 embryos (Fig 1E and (Doyle et al., 1989)). In embryos devoid of terminal activity, *zen* expression is not sustained at the embryonic poles (Helman et al., 2012); this was the case for all the embryos laid by $clos^{1}$ females at all the temperatures tested (Fig. 1B and 1F). However, in some cases, when the females were raised at 29ºC, we observed a clear ventral expansion of *zen* (Fig. 1G), revealing an expansion of the dorsal fate, which is in agreement with the cuticle phenotype. In addition, we observed a genetic interaction between *ndl* and *clos* in dorsoventral patterning, as the penetrance of the dorsoventral phenotype in embryos from $clos^{1}$ females increased in a *ndl* hemyzigous background (Table1).

Ndl protease activity is impaired in *fs(1)N*, *fs(1)ph* and *clos* mutants

The dorsoventral phenotype and the eggshell integrity defects of *clos*^{1} mutants and its sensitivity to the dose of *ndl* prompted us to examine whether Nasrat, Polehole and Clos are required for Ndl function. During mid-oogenesis, Ndl is secreted as a zymogen of 350 kDa, which is cleaved in late oogenesis to generate a C-terminal polypeptide of 250 kDa containing the Ndl protease domain (LeMosy et al., 1998) Fig2A). We detected the C-terminus fragment of Ndl both in egg chambers from wild-type and from *fs(1)N* null mutant females (Fig. 2B)., the latter also lacking Nasrat and Clos accumulation at the vitelline membrane (Jimenez et al., 2002); (Ventura et al., 2010). Later on, in embryogenesis, Ndl is further processed by at least two proteolytic events. The first, occurring at egg activation, is independent of Nudel protease activity, and it generates a 110-130-kDa C-terminal polypeptide from the 250 kDa fragment (LeMosy and Hashimoto, 2000). The second one depends on Nudel protease activity itself and gives rise to a 50-60-kDa C-terminal polypeptide from the C-terminal 110-130 kDa polypeptide (LeMosy et al., 1998) and Fig2A). We could detect the same proteolytic pattern although, in our hands, we observed slightly different molecular weights. Accordingly, Western blots from wild-type embryos (Fig. 2C lane 1) showed a C-terminal 50-60 kDa doublet, which is generated by Ndl-dependent protease activity as it is not detected in embryos derived from *ndl*^{111} mutant females with compromised Ndl protease activity (LeMosy et al., 2000) (Fig. 2C lane 2 and (LeMosy et al., 2000). Neither was the Ndl C-terminal 50-60 kDa doublet detected in embryos from *fs(1)N* null mutant females (Fig. 2C lane 3), thereby indicating impaired Ndl protease activity. Of note, in embryos from either *ndl*^{111} or *fs(1)N* null mutant females, the impaired Ndl protease activity caused the accumulation of intermediate C-terminal polypeptides (110-130kDa) generated in a Ndl-independent way. These results suggest that the failure in Ndl protease activity detected in the embryos laid by *fs(1)N* null mutant females accounts for the eggshell integrity phenotype observed in *fs(1)N*, *fs(1)ph* and *clos* null mutant females, although it may not be its only cause. Furthermore, we found a correlation between the dorsalised phenotype and the eggshell integrity defects in embryos from *clos*^{1} females and the activity of Ndl protease. Thus, we unveil a lack of proper Ndl protease activity in embryos laid by *clos*^{1} mothers raised at 29ºC as compared with those laid by *clos*^{1} mothers raised at 18ºC (Fig. 2D compare lanes 2 and 3). These observations strongly suggest that the dorsalised phenotype and the eggshell

integrity defects in embryos laid by *clos*^{1} females at 29ºC are caused by the lack of Ndl proteolytical activity.

Ndl localisation is impaired in *fs(1)N*, *fs(1)ph* and *clos* mutants

As mentioned above, Nasrat, Polehole and Clos proteins are required either for the anchoring or stabilization of Tsl at the vitelline membrane (Jimenez et al., 2002) (Ventura et al., 2010); (Stevens et al., 2003). Therefore, we examined whether this could also be the case for Ndl. Indeed, we found that secreted Ndl overlaps with the vitelline membrane component Polehole (Fig3A-A') but not with a protein of the oocyte plasma membrane such as the yolk receptor Yolkless (Yl) (Schonbaum et al., 2000) (Fig. 3B-B'), thereby indicating that during oogenesis Ndl accumulates at the vitelline membrane once secreted from the follicle.

Furthermore, we found that the proper accumulation of Ndl at the vitelline membrane depends on Nasrat, Polehole and Clos proteins. In particular, in stage 9 egg chambers from null *fs(1)ph* mutant females, Ndl was partially mislocalised inside the oocyte (Fig. 3C-D'), thereby suggesting that the vitelline membrane does not hold or stabilize all the Ndl protein in the absence of Nasrat, Polehole and Clos. The non-localized Ndl protein may be degraded and/or eliminated, as we did not detect it at stage 10 (Fig. 3E-F'). However, again, we detected an abnormal pattern at stages 12-14, when in the wild-type egg chambers, Ndl accumulated between follicle cells in aggregates together with other vitelline membrane proteins (data not shown; (Furriols and Casanova, 2014b); conversely, in around 70% of *fs(1)ph* mutant females, Ndl showed a lumpy distribution between follicle cells and the oocyte plasma membrane (Fig. 3G-H').

Since we found that Ndl anchorage or stabilisation at the vitelline membrane partially depends on Nasrat, Polehole and Closca, we questioned whether this was due to a specific role of these proteins on Ndl. To address this point, we first examined whether structural vitelline membrane components were affected in egg chambers from *fs(1)N* mutant mothers and found this not to be the case as sV23 (Fig. 4A) and VM32E (data not shown) localised correctly in these chambers. We next examined Ndl localisation in egg chambers from *Vm26Ab*^{QJ42} mutant mothers, which lack the sV23 protein and lay eggs that collapse due to defects in vitelline membrane integrity (Savant and

Waring, 1989). We found that Ndl, as well as Polehole and Tsl, localised correctly at the vitelline membrane (Fig. 4B-D). Finally, while Nasrat, Polehole and Clos were found to be required for Ndl localisation, the opposite was not true, as Polehole and also Tsl localised correctly in egg chambers from *ndl* null flies (fig4E-F). All together, these observations suggest that Nasrat, Polehole, Clos have a specific role in Ndl stabilisation or localisation at the vitelline membrane.

Discussion

Terminal and dorsoventral signalling rely on initial spatial cues originated in the follicle cells surrounding the oocyte that induce pattern formation in embryogenesis. Since follicle cells degenerate long before the cues perform their action in embryogenesis, all the information necessary for embryonic patterning has to be retained in the egg. In this scenario, the role of Nasrat, Polehole and Clos in the localisation of Tsl and Ndl suggests that a Nasrat/Polehole/Clos complex acts as a multifunctional hub at the vitelline membrane to anchor various proteins synthesized at oogenesis and with later functions in the eggshell and/or in triggering embryonic patterning.

Although the mechanism responsible for eggshell integrity is not fully understood, Nasrat, Polehole and Clos clearly participate in this process (Cernilogar et al., 2001). Moreover, Ndl, and in particular its protease activity, is also required for eggshell integrity (LeMosy and Hashimoto, 2000). Our results now identify Ndl as an effector of Nasrat, Polehole and Clos both in eggshell integrity and in their so far unknown role in dorsoventral patterning. In this regard, it is worth mentioning that, in spite of the many analyses of Ndl activity, it remains an open question as to whether its function in dorsoventral axis specification and eggshell integrity are independent of each other (LeMosy and Hashimoto, 2000).

Likewise, it is difficult to establish whether the diverse roles of Nasrat, Polehole and Clos imply specific and independent functional protein domains. Although Nasrat, Polehole and Clos belong to a common group of proteins, they show only moderate similarity, and no functional domains have been identified in any of them (Jimenez et

al., 2002; Ventura et al., 2010). The observation that a point mutation impairs the terminal functions of Clos proteins, as well as dorsoventral patterning and also eggshell integrity, suggests a lack of clear independent domains responsible for each individual function. However, *fs(1)N*^{211}and *fs(1)ph*^{1901} mutants are thought to specifically impair the terminal function of Nasrat and Polehole proteins, respectively, which suggests that these might be modular proteins with different functional domains. Similarly, the *fs(1)N*^{A1038} mutation supports the notion of independent functional domains (Degelmann et al., 1990). In particular, all eggs from homozygous *fs(1)N*^{A1038} females collapse due to eggshell integrity defects; the same phenotype is observed in hemyzigous *fs(1)N*^{A1038} females and in transheterozygote females of *fs(1)N*^{A1038} over a null *fs(1)N* mutant allele (Degelmann et al., 1990). However, eggs from transheterozygous females of *fs(1)N*^{A1038} over the *fs(1)N*^{211} terminal allele give rise to *wild-type* larvae and adults (Degelmann et al., 1990) and our own observations). This intra allelic complementation suggests that separate domains specifically affect the integrity and the terminal functions of the Nasrat protein. To further characterize these putative protein domains, we mapped the molecular lesion in the *fs(1)N*^{A1038} mutation and found it to correspond to an E to V transition at residue 350 (data not shown). This observation suggests that the domain of the Nasrat protein encompassing this residue is required for eggshell integrity but has no effect on embryonic patterning.

In conclusion, we have found that terminal and dorsoventral signalling, hitherto considered independent in their extracellular pathways, have Nasrat, Polehole and Clos as common mediators. We propose that a complex of these proteins constitutes a multifunctional hub to ensure the proper temporal localisation/stabilisation and activity of proteins synthesised at oogenesis and required at egg activation and to guarantee the coordination of the hardening of the eggshell with the trigger of early embryonic patterning.

Materials and Methods

Fly stocks

We used the following *Drosophila* stocks described in Flybase: *yw* flies as *wild type*, *fs(1)Ph-HA, Def(3L)CH12*, $fs(1)ph^{K646}$, $fs(1)N^{14}$, $clos^{1}$, $clos^{4152}$, ndl^{14}, ndl^{111}, $fs(1)N^{A1038}$, $Vm26Ab^{QJ42}$, *Def BSC183*.In all the experiments we employed the following transheterozygous combinations: $clos^{1}/clos^{4152}$, ndl^{111}/*Def(3L)CH12,* ndl^{14}/*Def(3L)CH12,* $Vm26Ab^{QJ42}$ / *Def BSC183*. $clos^{4152}$, ndl^{14} and $Vm26Ab^{QJ42}$ are null alleles of *clos, ndl* and *Vm26Ab.* The *Def BSC183* and *Def(3L)CH12* uncover the *Vm26Ab* and *ndl* loci respectively.

Antibodies and Immunostainings

For cuticle preparations, embryos were dechorionated with bleach, washed with 0.1% Triton, mounted with Hoyer:lactic (1:1), and incubated at 50–60°C overnight. At least 150 embryos were scored for cuticle preparations. Whole-mount *in situ* hybridisations were perfomed according to (Tautz and Pfeifle, 1989) with minor modifications. Embryos were hybridized overnight at 56ºC with antisense probes labeled with digoxigenin (DIG). Immunostainings were done as described in (Furriols and Casanova, 2014b)Egg chambers for Tsl immunostainings were heat-fixed as described in (Mineo et al., 2015). The following primary antibodies were used: anti-Tsl 1:50 (Grillo et al., 2012), anti-Yl 1:1/500 (Schonbaum et al., 2000), anti-HA 1/300 (3F10 Roche), anti-Spectrin 1/5 (3A9, D.S.H.B.), anti-sV23 1/100 (Pascucci et al., 1996), anti C-Ndl 1:400 (LeMosy et al., 1998), Anti-DIG (Roche; 1:200 and secondary antibodies 1/300 (Jackson ImmunoResearch). Confocal images were obtained with a Leica SPE, analysed in Fiji and assembled with Adobe Photoshop.

Western blot analysis

Ovaries were dissected in cold 1X PBS solution and immediately homogenised. Embryos were collected on apple juice plates and washed extensively with water. Samples were homogenized on ice in 2XSDS sample buffer containing 6 M urea and 100 mM DTT accordingly to (LeMosy et al., 1998), boiled for 5 minutes and soluble proteins loaded on 10% or 4-20% SDS-polyacrylamide gels (Amersham). Typically, 3 ovaries or 50-60 embryos from 0-1,5h were loaded per lane. Proteins were transferred to PVDF membranes (Millipore), blocked with Odissey blocking buffer PBS (Licor) and incubated with primary antibodies overnight. Membranes were analised using the

Odyssey CLx imaging system (Licor). The following antibodies were used: anti C-Ndl 1:1000 (LeMosy et al., 1998), anti-Tubulin 1:5000 (Millipore) and secondary antibodies 1/10000 (IRDye 800 and 680 from Licor).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.M. and M.F. performed the experiments. A.M., M.F. and J.C. conceived and designed the experiments, analysed the data and wrote the paper.

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Table 1: percentage of dorsalised phenotypes

	clos^{1}/clos^{4152}	*clos^{1}/clos^{4152}; ndl/+*
18ºC	**1%**	**8%**
25ºC	**7%**	**41%**
29ºC	**45%**	**79%**

Figures and Figure Legends

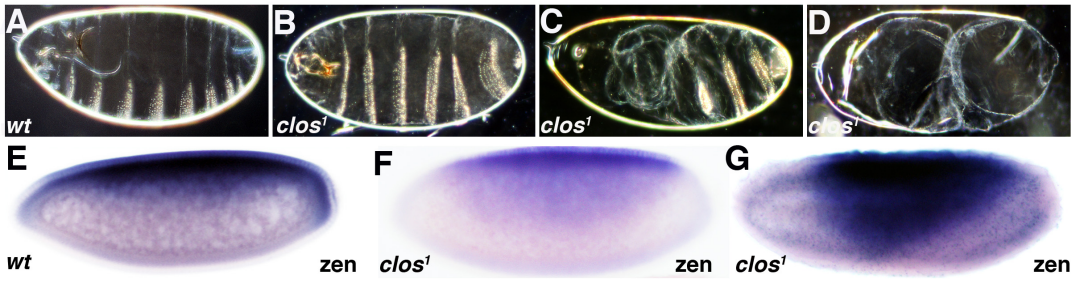

Fig1. *clos*^{1} mutants show defects in dorsoventral patterning

(A) Cuticle of a wild type embryo. (B-D) Cuticles of embryos derived from *clos*^{1}/*clos*^{4152} females raised at 29ºC. (B) Cuticle of an embryo displaying a terminal phenotype (note the lack of all anterior and posterior terminal structures). (C) Cuticle of an embryo displaying a terminal phenotype and a partially dorsalised phenotype (note the lack of the posterior terminal structures and the lack of some ventral denticle belts anteriorly). (D) Cuticle of an embryo displaying a dorsalised phenotype (note the lack of the ventral denticle belts). (E-G) *In situ* hibridisation of *zerknullt* (*zen*) in a *wild type* embryo (E) and in embryos derived from *clos*^{1}/*clos*^{4152} raised at 29ºC (F-G). In these embryos, *zen* mRNA expression is not sustained at the poles reflecting lack of Tor activation (F-G) and, in some cases, it also expands ventrally reflecting a lack of Toll activation (G).

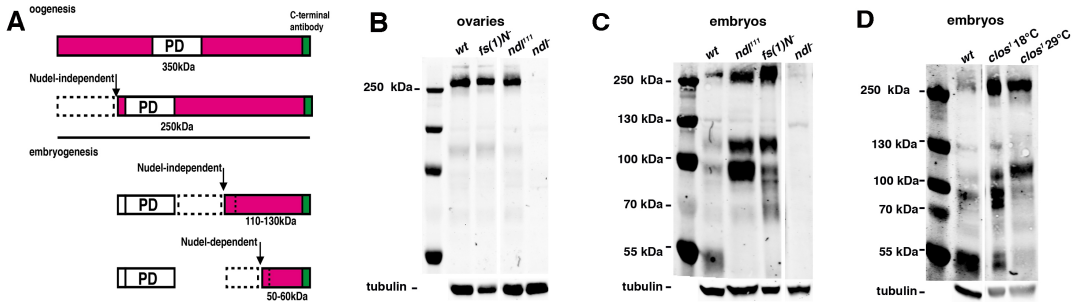

Fig2. **Nasrat, Polehole and Closca are required for Ndl activity**

(A) Schematic diagram of Nudel processing defined by Western blots adapted from (LeMosy et al., 1998). PD indicates the Ndl protease domain, Ndl C-terminal polypetides in purple, the region of Ndl recognised by the C-terminal polyclonal antibody in green (B) C-terminal Ndl polypeptides in ovaries from *wt, fs(1)N*^{14}, *ndl*^{111}/*Def(3L)CH12* and *ndl*^{14}/*Def(3L)CH12* females. 350 kDa Ndl (at very low

intensity) and 250kDa Ndl C-terminal polypeptide are detected in all cases except in ovaries derived from *ndl*^{14}*/Def(3L)CH12* females. *ndl*^{14}(lane 4) encodes for a truncated Ndl protein that is not recognised by the antibody (LeMosy et al., 2000). (C) C-terminal Ndl polypeptides in embryos from *wt, fs(1)N*^{14}*, ndl*^{111}*/Def(3L)CH12* and *ndl*^{14}*/Def(3L)CH12* females. Embryos from *fs(1)N*^{14} and *ndl*^{111} females (lane 2-3) completely lack the 50–60 kDa C-terminal polypeptides which require functional Nudel protease activity. Embryos from *fs(1)N*^{14} and *ndl111* females (lane2-3) show increased levels of two Ndl C-terminal polypeptides of around 100kDa, which are the precursors of the 50-60 kDa polypetides; compare to *wild type* (lane1). (D) C-terminal Ndl polypeptides in *wild type* embryos and in embryos from *clos*^{1}*/clos*^{4152} females raised at 18°C and at 29°C. High levels of he 50–60 kDa processed C-terminal polypeptides are detected in embryos from *wt* and *clos*^{1}*/clos*^{4152} females raised at 18°C (lane 1-2) but are strongly reduced in embryos from *clos*^{1}*/clos*^{4152} females raised at 29°C (lane 3). High levels of of the Ndl C-terminal polypeptides of around 100kDa are detected in embryos laid by *clos*^{1}*/clos*^{4152} females (lanes 2- 3) compared to the *wt* (lane 1). In all cases, an antibody against Tubulin 50kDa (bottom) was used for a loading control.

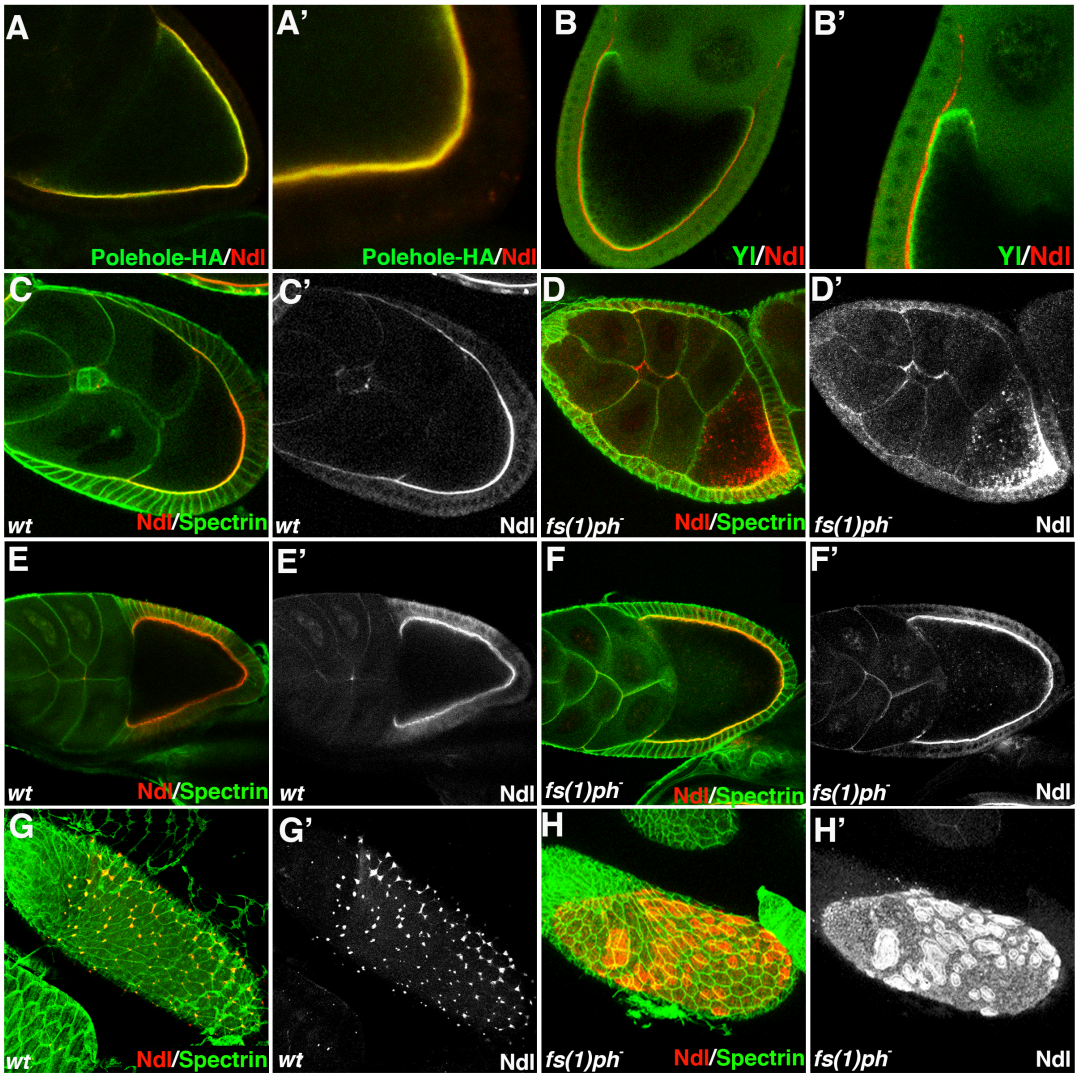

Fig3. **Ndl localise at the vitelline membrane anchored or stabilised by Nasrat, Polehole and Closca**

(A) Confocal section of a stage 9 egg chamber expressing Polehole-HA and immunostained with anti-C-Ndl (in red) and anti-HA(in green) antibodies. (A′) A magnification of A showing the colocalisation. (B) Confocal section of a stage 9 egg chamber immunostained with anti-C-Ndl (in red) and anti-Yl (in green) antibodies. (B′) A magnification of B showing the lack of colocalisation. (C-H) Confocal sections of wt and *fs(1)ph*^{K646} egg chambers at different stages immunostained with anti-C-Ndl (in red) and anti-Spectrin (in green) antibodies. (C,D) Stage 9; (E,F) Stage 10; (G-H) stage 12. (C′-H′) Same images in the red channel. Note mislocalised Ndl inside the oocyte at

stage 9 (D,D´) and the Ndl lumpy distribution at stage 12 (H-H´) in *fs(1)ph*^{K646} egg chambers .

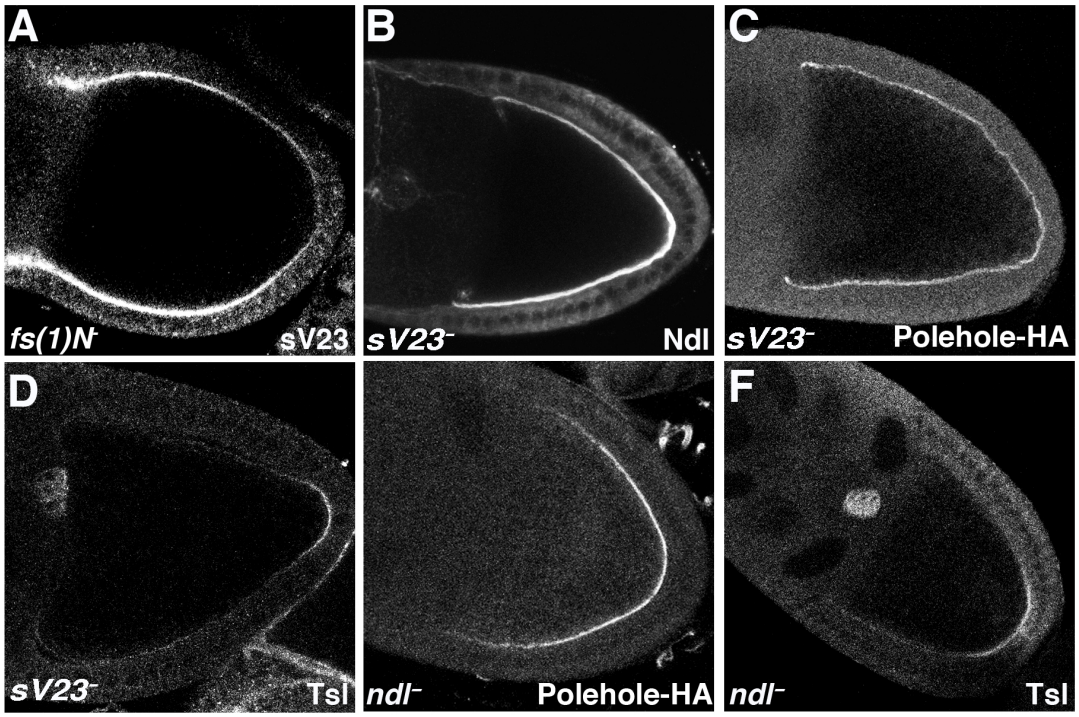

Fig.4 Accumulation of vitelline membrane components in mutant egg chambers. (A) Confocal section of a *fs(1)N*^{14} stage 9 egg chamber showing a wild-type accumulation of sV23 as detected by the anti-sV23 antibody. (B-D) Confocal sections of *Vm26Ab*^{QJ42}*/DefBSC183* stage 9 (B,D) or early stage 10A (C) egg chambers, which lack sV23 protein, immunostained with anti-C-Ndl antibody (B), expressing the Polehole-HA and immunostained with anti-HA antibody (C) and immunostained with anti-Tsl antibody (D). In all three cases there is a *wild-type* pattern. (**E,F**) Confocal sections of *ndl*^{14}*/Def(3L)CH12* stage 9 egg chambers expressing the Polehole-HA and immunostained with anti-HA antibody (E) and immunostained with anti-Tsl antibody (F). In both cases, there is a *wild-type* pattern

Appendix II

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RESEARCH REPORT

Accumulation of the *Drosophila* Torso-like protein at the blastoderm plasma membrane suggests that it translocates from the eggshell

Alessandro Mineo, Marc Furriols* and Jordi Casanova*

ABSTRACT

The eggshell serves as a depository for proteins that play an important role in early embryonic development. In particular, the *Drosophila* eggshell is responsible for transferring asymmetries from the egg chamber to specify the regions at both ends of the embryo through the uneven activation of the Torso (Tor) receptor in its membrane. This process relies on the restricted expression of the gene *torso-like* (*tsl*) in subpopulations of follicle cells during oogenesis and its protein accumulation at both poles of the eggshell, but it is not known how this signal is transmitted to the embryo. Here, we show that Tsl accumulates at the embryonic plasma membrane, even in the absence of the Tor receptor. However, during oogenesis, we detected Tsl accumulation only at the eggshell. These results suggest that there is a two-step mechanism to transfer the asymmetric positional cues from the egg chamber into the early embryo: initial anchoring of Tsl at the eggshell as it is secreted, followed by its later translocation to the egg plasma membrane, where it enables Tor receptor activation. Translocation of anchored determinants from the eggshell might then regulate the spatial and temporal control of early embryonic developmental processes.

KEY WORDS: *Drosophila*, MACPF domain, Plasma membrane, Torso, Torso-like, Vitelline membrane

INTRODUCTION

The *Drosophila* ovary provides the positional cues that specify the pattern of the future embryonic body plan. In particular, the transduction pathways specifying the dorso-ventral axis and the two embryonic end regions rely on asymmetries between the ovarian cells for the locally restricted activation of their corresponding receptors (reviewed by Stein and Stevens, 1991). In the case of the terminal regions, these are specified by the activity of the Torso (Tor) receptor tyrosine kinase, which is present throughout the entire blastoderm membrane but activated only at the poles. The precise mechanism of Tor activation is unknown, but it depends on the expression of *torso-like* (*tsl*) in subpopulations of follicle cells at both ends of the oocyte. In embryos from *tsl* mutant females, the Tor receptor is not activated and, conversely, ubiquitous expression of *tsl* in follicle cells leads to general activation of the Tor receptor throughout the embryo (reviewed by Furriols and Casanova, 2003).

Given that the *tsl*-expressing cells degenerate at the end of oogenesis and are not present at the time the Tor receptor is activated at early embryogenesis (Schupbach and Wieschaus, 1986; Sprenger et al., 1993), there must be a mechanism to ensure the transfer of the asymmetric positional cues from the egg chamber to the early embryo (Casanova and Struhl, 1993). This mechanism appears to be linked to eggshell proteins. A first indication pointing to this link came from the discovery that *fs(1)Nasrat* [*fs(1)N*] and *fs(1)polehole* [*fs(1)ph*; *fs(1)M3* – FlyBase], two germline genes required for eggshell biogenesis, have hypomorphic mutations that do not affect eggshell formation but prevent Tor receptor activation (Degelmann et al., 1990).

A further confirmation of this link came from the analysis of Tsl protein distribution (Stevens et al., 2003). Although Tsl was initially reported to accumulate at the embryonic poles, an observation taken as an indication consistent with Tsl being the ligand of the Tor receptor (Martin et al., 1994), it was not possible to replicate this result (Stevens et al., 2003). Instead, later experiments found that Tsl accumulated in laid eggs at the internal side of the vitelline membrane, the innermost layer of the eggshell (Stevens et al., 2003). Interestingly, Tsl extracellular accumulation is dependent on the *fs(1)N*, *fs(1)ph* and *closca* genes, indicating that the localisation of Tsl depends on eggshell proteins secreted from the oocyte (Jiménez et al., 2002; Stevens et al., 2003; Ventura et al., 2010). However, it remained an open question as to how the accumulation of Tsl at the eggshell could influence the embryo. Furthermore, the finding that Tsl harbours a membrane-attack complex/perforin domain (MACPF) (Ponting, 1999), a domain present in proteins involved in the formation of pores at the plasma membrane (for a review see Voskoboinik et al., 2006), made this observation difficult to reconcile with a function at the eggshell.

Many data have pointed alternatively to the trunk (*trk*) protein being the Tor ligand. *trk* RNA accumulates in the oocyte and its protein is likely to be secreted into the perivitelline fluid between the embryo and the eggshell (Casanova et al., 1995). Trk shares structural features with growth factors and its C-terminal fragment activates the Tor pathway even in a *tsl* mutant background (Casali and Casanova, 2001); however, Tsl still requires *trk* function to activate the Tor pathway (Furriols et al., 1998). These results prompted the notion that Tsl is involved in the cleavage of Trk, which would then behave as the ligand for the Tor receptor (Casanova et al., 1995). However, this notion for Tsl function has recently been challenged (Henstridge et al., 2014) (and see below).

Here, using a new anti-Tsl antibody (Grillo et al., 2012) and an alternative fixation procedure, we show that Tsl accumulates at the blastoderm plasma membrane, even in the absence of the Tor receptor, thereby ruling out that this accumulation is indicative of Tsl being a Tor ligand (Martin et al., 1994). Furthermore, during oogenesis, we detected Tsl accumulation only at the vitelline membrane. These results suggest that there is a two-step mechanism through which the Tsl asymmetric positional cue is transferred from the egg chamber into the early embryo: initial anchoring of Tsl at the

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vitelline membrane as the protein is secreted by the follicle cells, followed by its later translocation to the oocyte plasma membrane, where it would enable Tor receptor activation.

RESULTS AND DISCUSSION

By means of a haemagglutinin-tagged Tsl construct (Tsl-HA), we previously reported that Tsl is found in the cytoplasm of a specialised group of follicle cells surrounding the oocyte and extracellularly between the follicle cells and the oocyte (Jiménez et al., 2002). We have now been able to generate an anti-Tsl antibody (Grillo et al., 2012) that reproduces the previously unveiled Tsl-HA pattern (Fig. 1A). We have further proved the specificity of the antibody by the absence of signal in females homozygous for tsl^{604} (Fig. 1B), a P-element-induced mutation in the promoter that eliminates *tsl* function (Martin et al., 1994).

Nevertheless, this antibody was unable to reveal any specific signal in embryos upon standard chemical fixation or by electron microscopy (data not shown). However, when embryos were instead subjected to heat-fixation (Tepass, 1996; Tanentzapf et al., 2007), we detected a clear Tsl accumulation at both embryonic poles (Fig. 1C-E), which was also specific to Tsl because it was absent in embryos from tsl^{604} females (Fig. 1F) and was found throughout the embryo upon ectopic expression of *tsl* in the follicle cells (Fig. 1G). Note that upon *tsl* ectopic expression, we also observed high levels of Tsl within embryos. We then analysed the requirements for Tsl accumulation at the embryonic poles. We first concluded that embryonic Tsl accumulation does not reflect receptor binding, as Tsl also accumulates in embryos without the Tor receptor (Fig. 1H). Similarly, Tsl accumulation at the embryonic poles was also independent of *trk* (Fig. 1I).

How can the previously reported accumulation of Tsl at the vitelline membrane in laid eggs be reconciled with Tsl accumulation at the plasma membrane? There is a time lapse between *tsl* expression in the follicle cells by stage 8 (Savant-Bhonsale and Montell, 1993; Martin et al., 1994) and Tor receptor accumulation in early embryogenesis (Schupbach and Wieschaus, 1986; Casanova and Struhl, 1993; Sprenger et al., 1993), when the follicle cells are no longer present. Our findings suggest that the mechanism that ensures that the spatial asymmetry in the ovary is transmitted to the embryo to allow restricted activation of the Tor receptor (Casanova and Struhl, 1989) and consists of the initial anchoring of Tsl, as it is secreted, into the vitelline membrane, followed by its later translocation to the embryonic plasma membrane.

To support this hypothesis, we then assessed the precise localisation of Tsl protein during oogenesis. Although Tsl-HA is detected extracellularly (Jiménez et al., 2002), the exact compartment of Tsl accumulation has never been analysed. We have found now that domain containing extracellular Tsl overlapped with that of the vitelline protein Nasrat (Fig. 2A). This observation is consistent with Tsl accumulation at the vitelline membrane in laid eggs (Stevens et al., 2003) and fits well with the requirement of Nasrat for Tsl extracellular accumulation (Jiménez et al., 2002; Stevens et al., 2003). As expected, Nasrat did not overlap with the Yolkless (Yl) receptor (Schonbaum et al., 2000) (Fig. 2B), an oocyte plasma membrane protein (we were unable to simultaneously examine the Tsl and Yl patterns as both antibodies are derived from rat). We obtained the same results for Polehole (Fig. 2C), a protein closely related to Nasrat (Jiménez et al., 2002). Of note, our experiments also showed variable patterns of vitelline membrane proteins, as determined by confocal microscopy. Thus, for example, the locations of Nasrat and VM32E, which have been assigned to the vitelline membrane by electron microscopy (Andrenacci et al., 2001) and identified as eggshell proteins by proteomics (Fakhouri et al., 2006), often do not overlap when viewed by confocal microscopy (Jiménez et al., 2002). This observation might indicate heterogeneity within the vitelline membrane or differences in antibody accessibility or recognition caused by sample processing and/or distinct fixation procedures used for confocal microscopy or for electron microscopy. Similarly, we also observe different patterns for Tsl and VM32E by confocal microscopy (Fig. 2D). In summary, these results point to the initial anchoring of Tsl at the vitelline membrane, which is probably mediated by interactions with the products of the *fs(1)N*, *fs(1)ph* and *closca* genes (Jiménez et al., 2002; Stevens et al., 2003; Ventura et al., 2010).

We then examined how early Tsl protein accumulates at the plasma membrane. As Tsl is detected in very young embryos (Fig. 2E), we addressed whether Tsl accumulation at the plasma membrane is linked to fertilisation; however, Tsl was also present in the plasma membrane of unfertilised eggs (Fig. 2F). Therefore, we conclude that Tsl accumulates at the plasma membrane between late oogenesis and fertilisation. In this period, the most prominent changes are related to egg activation, the conversion of the oocyte into an egg capable of supporting embryogenesis (Sartain and Wolfner, 2013). Although in higher organisms fertilisation is coupled to egg activation (Sartain and Wolfner, 2013), in *Drosophila*, as in many insects, these two events are independent, and egg activation takes place as the oocytes pass through the oviducts before becoming fertilised (Heifetz et al., 2001). While passing through the oviduct, eggs swell, causing an increase in their Ca^{2+} concentration, which in turn triggers many physiological events including resumption of meiosis of the female pronucleus, translation of maternal mRNAs and crosslinking of the vitelline membrane (Sartain and Wolfner, 2013).

However, a direct analysis of Tsl accumulation at the oocyte plasma membrane just before egg activation could not be performed because it is not technically possible to obtain properly heat-fixed eggshell-free oocytes. We also reproduced the egg activation process *in vitro* (Endow and Komma, 1997; Page and Orr-Weaver, 1997) but were not able to readily separate the oocytes from their vitelline membranes. Finally, we examined mutants for *sarah* (*sra*), the gene encoding an inhibitor of calcineurin, a Ca^{2+}-dependent phosphatase, in which egg activation begins but does not progress. In *sra* mutants many events of egg activation fail, but the cross-linking of the vitelline membrane components is apparently normal (Horner et al., 2006; Takeo et al., 2006). Eggs from *sra* females (see Materials and Methods) showed Tsl accumulation in plasma membranes (Fig. 2G), thus indicating that this process is not dependent on *sra*.

We also examined whether any of the existing *tsl* point mutations specifically impairs a particular step in Tsl accumulation. For tsl^{1}, tsl^{2} and tsl^{3} and tsl^{5} point mutants (Savant-Bhonsale and Montell, 1993), we found that Tsl protein accumulated at the plasma membrane (data not shown), indicating that these mutations render the protein non-functional without interfering with its secretion, anchorage or translocation. Of note, we observed a reduction in Tsl levels at the plasma membrane in tsl^{3} mutants; however, tsl^{3} does not appear to specifically affect accumulation of Tsl at the plasma membrane as the protein fails to remain localised to the vitelline membrane in tsl^{3} mutants (unpublished results in Stein et al., 2008). Besides its role in terminal patterning, *tsl* plays an additional role in regulating timing at pupariation (Grillo et al., 2012; Johnson et al., 2013). In this regard, Tsl-HA can replicate *tsl* function to regulate the timing of pupariation but cannot function in terminal patterning (Johnson et al., 2013). We thus examined the accumulation of Tsl-HA and found that, in spite of properly accumulating at the egg

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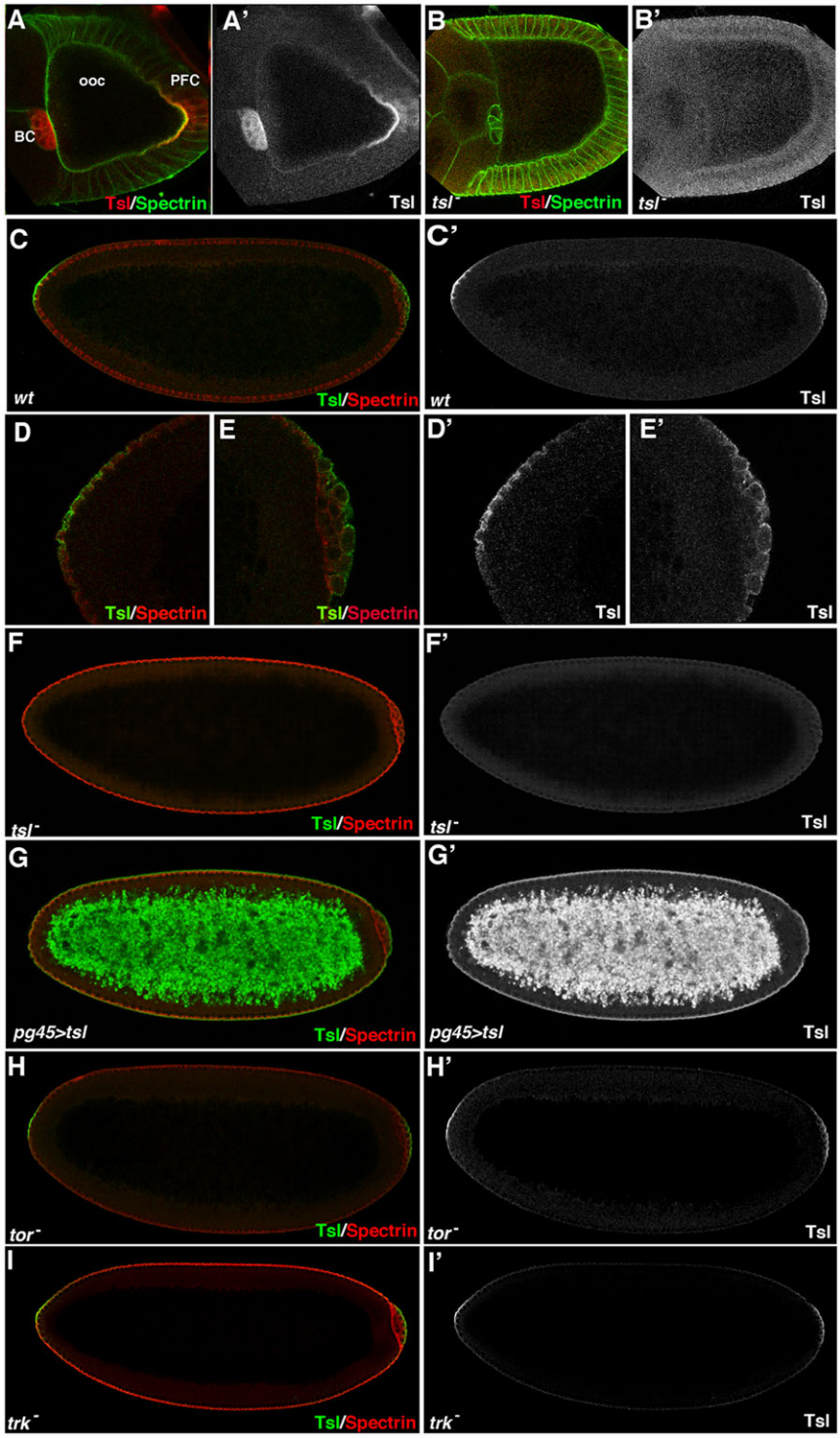

Fig. 1. Torso-like accumulates at the poles of the embryonic plasma membrane. (A) Confocal section of a stage 10 egg chamber. (A′) Same image in the red channel; Tsl is detected in border cells (BC), posterior follicle cells (PFC) and extracellularly between the oocyte (ooc) and the follicle cells. (B) Confocal section of a tsl^{604} stage 10 egg chamber. (B′) Same image in the red channel. The gain is enhanced in the confocal microscopy to make the absence of Tsl clearer. (C) Projection of confocal sections of a blastoderm. (D,E) Magnifications at the anterior D and the posterior poles E. (C′-E′) Same images as C-E in the green channel. (F) Projection of confocal sections of a tsl^{604} blastoderm. (F′) Same image in the green channel. (G) Projection of confocal sections of a blastoderm from a *tsl*-overexpressing female. (G′) Same image in the green channel; Tsl is around all of the plasma membrane. Note the high levels of Tsl inside the embryo. (H) Projection of confocal sections of a tor^{XR} blastoderm. (H′) Same image in green channel. (I) Projection of medial confocal sections of a trk^{RI53} blastoderm. (I′) Same image in the green channel. (C-I) Anti-Tsl antibody immunostaining is shown in green and anti-Spectrin immunostaining in red.

chamber, where it overlapped with the Viteline membrane-like (Vml) protein (Zhang et al., 2009) (Fig. 3A,B), it failed to accumulate in the embryonic plasma membrane (Fig. 3E). We confirmed these results with the anti-Tsl antibody, which recognised Tsl-HA in the ovaries (Fig. 3C,D) but not at the embryonic plasma membrane (Fig. 3F). These results are thus consistent with a functional relevance for Tsl accumulation at the plasma membrane for terminal patterning.

The plasma membrane accumulation of Tsl clearly fits with it harbouring an MACPF domain (Ponting, 1999), which is present in proteins that associate with the plasma membrane to form membrane pores (for a review, see Voskoboinik et al., 2006) and, particularly interesting for the Tor pathway, are involved with the delivery of proteolytic enzymes (Bolitho et al., 2007). As a cleaved form of Trk activates the Tor receptor, bypassing Tsl function (Casali and Casanova, 2001), the notion has emerged that Tsl is involved in the specific cleavage of Trk (Casanova et al., 1995). However, MACPF proteins are usually involved in the entry of enzymes for intracellular proteolysis, whereas the Trk cleavage is thought to occur extracellularly. Indeed, Trk has been recently

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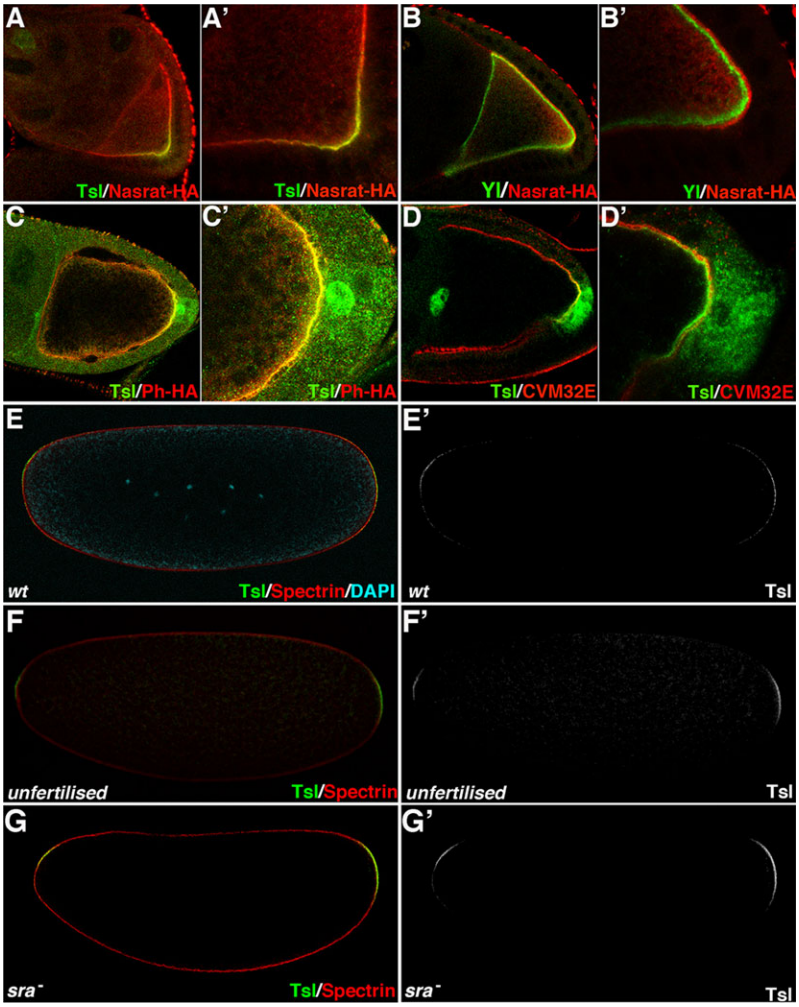

Fig. 2. Torso-like accumulation in development. (A) Confocal section of a stage 9 egg chamber expressing Nasrat-HA and immunostained with anti-Tsl and anti-HA antibodies. (A′) A magnification of A showing the colocalisation. (B) Confocal section of a stage 9 egg chamber expressing Nasrat-HA immunostained with anti-HA and anti-Yl antibodies. (B′) A magnification of B showing their different patterns. (C) Confocal section of a stage 10 egg chamber expressing Polehole-HA immunostained with anti-Tsl and anti-HA antibodies. (C′) A magnification of C showing their colocalisation. (D) Confocal section of a stage 10 egg chamber immunostained with anti-Tsl and anti-cVM32E antibodies. (D′) A magnification of D showing their different patterns. (E) Projection of confocal sections of a third mitotic division embryo immunostained anti-Tsl and anti-Spectrin antibodies and DAPI. (E′) Same image as E in the green channel. (F) Projection of confocal sections of an unfertilised egg immunostained with anti-Tsl and anti-Spectrin antibodies. (F′) Same image as F in the green channel. (G) Projection of confocal sections of an embryo from an sra^{A426}/sra^{KO} female immunostained with anti-Tsl and anti-Spectrin antibodies. (G′) Same image as G in the green channel.

shown to go through multiple proteolytic cleavages, although in a *tsl*-independent manner (Henstridge et al., 2014). Whether there is an as yet unidentified proteolytic step that is indeed *tsl*-dependent or whether Tsl is involved in a different process, the fact remains that a cleaved form of Trk produced in the oocyte does not require *tsl* function to activate the Tor receptor, whereas the full-length Trk protein does (Furriols et al., 1998; Casali and Casanova, 2001). It is also worth noting that anchorage of MACPF proteins depends on Ca^{2+} (Voskoboinik et al., 2005), which is also required for egg activation (Sartain and Wolfner, 2013). Thus a change in Ca^{2+}

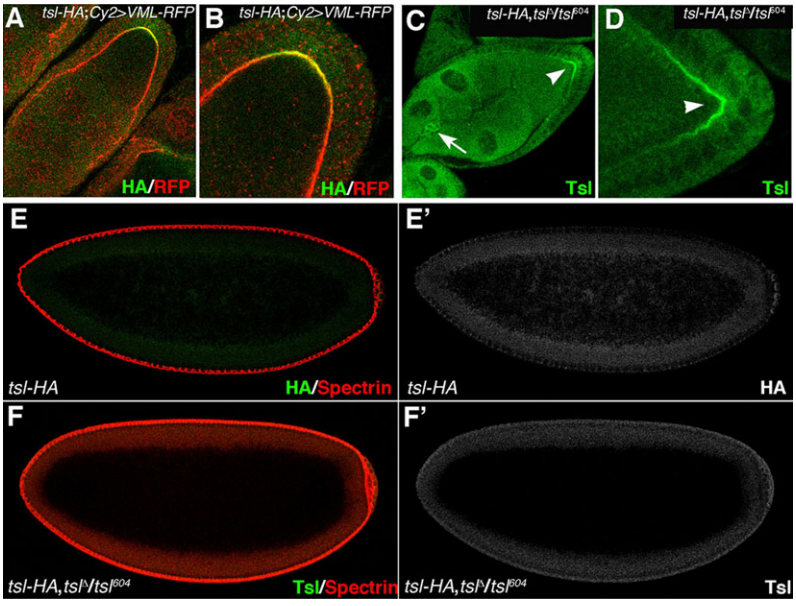

Fig. 3. Tsl-HA fails to accumulate at the embryonic plasma membrane. (A) Confocal section of a stage 9 egg chamber expressing Tsl-HA and UAS-RFP-VML under the Cy2Gal4 driver immunostained anti-HA and anti-RFP antibodies. (B) A magnification of A showing the colocalisation. (C) Confocal section of a tsl^{Δ}/tsl^{604} stage 9 egg chamber expressing Tsl-HA; anti-Tsl antibody immunostaining shows its normal accumulation in the border cells (arrow) and extracellularly at the posterior pole (arrowhead). (D) Confocal section of a similar stage 9 egg chamber showing the extracellular Tsl-HA accumulation in more detail (arrowhead). (E) Projection of confocal sections of a blastoderm expressing Tsl-HA immunostained with anti-HA and anti-Spectrin antibodies. (E′) Same image as E in the green channel. (F) Projection of confocal sections of a tsl^{Δ}/tsl^{604} blastoderm expressing Tsl-HA, immunostained with anti-Tsl and anti-Spectrin antibodies. (F′) Same image as F in the green channel.

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concentration might act to trigger Tsl translocation, linking it to egg activation and eggshell crosslinking (Fig. 4). It would be interesting to sort out whether a translocation event could also occur for Nudel, a *Drosophila* protease secreted by the follicle cells and which is involved in eggshell crosslinking and dorsoventral patterning (Hong and Hashimoto, 1995; LeMosy and Hashimoto, 2000). Although Nudel accumulates at the embryonic plasma membrane (LeMosy et al., 1998), it has been found at the oocyte plasma membrane by confocal microscopy (LeMosy et al., 1998) but is defined as an eggshell component by proteomics (Fakhouri et al., 2006).

It was proposed that Tsl anchoring in the vitelline membrane serves to keep Tsl restricted to the poles from oogenesis to early embryogenesis (Stevens et al., 2003). Our observations suggest that it acts to control developmental timing: Tsl accumulation at the plasma membrane and *tor* RNA translational control under a shared trigger (egg activation) should allow the simultaneous presence of Tsl and the Tor receptor at the embryonic plasma membrane, and thus the timely activation of the Tor pathway. Thus, translocation of determinants from the eggshell might serve as a general mechanism to provide spatial and temporal control of early embryonic developmental processes.

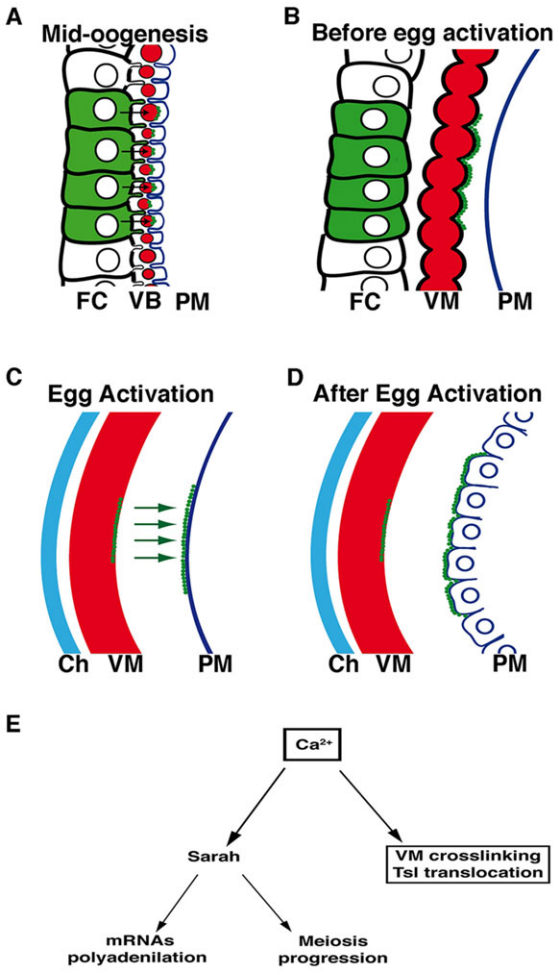

Fig. 4. A two-step model for the transfer of Tsl from follicle cells to the embryo. (A-D) Tsl is secreted from the follicle cells (FC) and incorporated into the vitelline bodies (VB), which fuse and form the vitelline membrane (VM). Upon egg activation, Tsl translocates to the oocyte plasmatic membrane (PM) and afterwards it is found both at the embryonic VM and PM. Ch, chorion. (E) Egg activation events. See text for details.

MATERIALS AND METHODS

Fly strains

The *Drosophila* stocks used are described in FlyBase. We used the heteroallelic combination sra^{A426}/sra^{KO}.

Antibodies and immunostaining

Heat fixation was as described previously (Tepass, 1996; Tanentzapf et al., 2007) with minor modifications (embryos were dechorionated for 2 min, E-wash solution was 100 mM NaCl, 0.3% Triton X-100 and cooling was by adding four volumes of ice-cold E-wash). Afterwards, embryos were devitellinized in heptane-methanol and collected in methanol. Ovaries were dissected as described previously (Furriols and Casanova, 2014) and heat fixed as described above. For *in vitro* egg activation, we followed published protocols (Endow and Komma, 1997; Page and Orr-Weaver, 1997). We used antibodies against Tsl 1/50 (Grillo et al., 2012), Yl 1/500 (Schonbaum et al., 2000), HA (12CA5 1/100 and 3F10 1/300, Roche), Spectrin (3A9, DSHB; 1/5), CVM32E 1/150 (Andrenacci et al., 2001), RFP (ab62341, Abcam; 1/300) and secondary antibodies (Jackson ImmunoResearch; 1/300). Confocal images were obtained with a Leica SPE microscope and analysed with Fiji software.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.M. and M.F. performed the experiments. A.M., M.F. and J.C. conceived and designed the experiments and analysed the data. J.C. wrote the paper.

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